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Sandra E. Marxen
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| UTILITY PATENT APPLICATION TRANSMITTAL UNDER 37 CFR §1.53(b) | |
|---|---|
| Attorney Docket Number | 50010/007006 |
| Applicant | Richard F Selden *et al.* |
| Title | *IN VIVO* PRODUCTION AND DELIVERY OF ERYTHROPOIETIN OR INSULINOTROPIN FOR GENE THERAPY |

**PRIORITY INFORMATION:**

This application is a continuation of and claims priority from United States Patent Application Serial No. 08/334,455, filed November 4, 1994, which is a continuation of U.S. Serial No. 07/911,533, filed July 10, 1992, which is a continuation-in-part of U.S. Serial No. 07/787,840, filed November 5, 1991, which is a continuation-in-part of U.S. Serial No. 07/789,188, filed November 5, 1991.

**APPLICATION ELEMENTS:**

| | |
|---|---|
| Cover sheet | [1] page |
| Specification | [54] pages |
| Claims | [38] pages |
| Abstract | [1] page |
| Drawings (Formal)<br>Petition To Accept Black & White Photographs<br>Transmittal of Formal Drawings to Official Draftsperson | [7] pages<br>[2] pages<br>[2] pages |
| Combined Declaration and POA, which is:<br>☐ Unsigned;<br>☐ Newly signed for this application;<br>☒ A copy from prior application 08/334,455 and the entire disclosure of the prior application is considered as being part of the disclosure of this new application and is hereby incorporated by reference therein. | [3] pages |
| Revocation and New Power of Attorney (as previously filed) | [3] pages |
| Transmittal of Substitute Declarations/Power of Attorney | [2] pages |
| Substitute Declaration for Patent Application | [3] pages |
| Sequence Listing on Paper | [3] pages |

| | |
|---|---|
| Small Entity Statement, which is:<br>☐ Unsigned;<br>☐ Newly signed for this application;<br>☒ A copy from prior application 08/334,455 and such small entity status is still proper and desired. | [2] pages |
| Preliminary Amendment | [2] pages |
| IDS | [**] pages |
| Form PTO 1449 | [**] pages |
| Cited References | [**] pages |
| Recordation Form Cover Sheet and Assignment | [**] pages |
| Assignee's Statement | [**] pages |
| English Translation | [**] pages |
| Certified Copy of Priority Document | [**] pages |
| Return Receipt Postcard | 1 |
| **FILING FEES:** | |
| Basic Filing Fee: $380 | $380.00 |
| Excess Claims Fee: 67 - 20 x $9 | $423.00 |
| Excess Independent Claims Fee: 9 - 3 x$39 | $234.00 |
| Multiple Dependent Claims Fee: $130 | $0.00 |
| Total Fees: | $1,037.00 |

☒ Enclosed is a check for $130.00 and $ 1,037.00 to cover the total fees.
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Signature Susan M. Michaud Reg. No. 42,885

June 8, 1999
Date

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Applicant or Patentee: Richard F Selden, Douglas Treco and Michael W. Heartlein Attorney's

Serial or Patent No.: 07/911,533 Docket No.: TKT91-01A

Filed or Issued: July 10, 1992

For: IN VIVO PRODUCTION AND DELIVERY OF ERYTHROPOIETIN OR INSULINOTROPIN FOR GENE THERAPY



VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS
(37 CFR 1.9(f) and 1.27(c) - SMALL BUSINESS CONCERN

I hereby declare that I am

[ ] the owner of the small business concern identified below:

[XX] an official of the small business concern empowered to act on behalf of the concern identified below:

NAME OF CONCERN Transkaryotic Therapies, Inc.

ADDRESS OF CONCERN 195 Albany Street
Cambridge, MA 02115

I hereby declare that the above identified small business concern qualifies as a small business concern as defined in 13 CFR 121.12, and reproduced in 37 CFR 1.9(d), for purposes of paying reduced fees to the United States Patent and Trademark Office, in that the number of employees of the concern, including those of its affiliates, does not exceed 500 persons. For purposes of this statement, (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the persons employed on a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third party or parties controls or has the power to control both.

I hereby declare that rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the invention, entitled IN VIVO PRODUCTION AND DELIVERY OF IRYTHROPOIETIN OR INSULINOTROPIN FOR GENE THERAPY by inventor(s) Richard F selden, Douglas Treco and Michael W. Heartlein described in

[ ] the specification filed herewith

[X] application serial no. 07/911,533, filed July 10, 1992.

[ ] patent no. _____, issued _____.

If the rights held by the above identified small business concern are not exclusive, each individual, concern or organization having rights to the invention is listed below* and no rights to the invention are held by any person, other than the inventor, who would not qualify as an independent inventor under 37 CFR 1.9(c) if that person made the invention, or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d), or a nonprofit organization under 37 CFR 1.9(e). *NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

NAME_____

ADDRESS_____

[ ] INDIVIDUAL [ ] SMALL BUSINESS CONCERN [ ] NONPROFIT ORGANIZATION

NAME_____

ADDRESS_____

[ ] INDIVIDUAL [ ] SMALL BUSINESS CONCERN [ ] NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING Richard F Selden, M.D., Ph.D.

TITLE OF PERSON OTHER THAN OWNER Chairman, Board of Scientific Advisors

ADDRESS OF PERSON SIGNING 195 Albany Street

Cambridge, MA 02115

SIGNATURE Richard F Selden DATE 08/7/92

PATENT
ATTORNEY DOCKET NO. 50010/007006

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Sandra E. Marxen — Printed name of person mailing correspondence

Sandra E Marxen — Signature of person mailing correspondence

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

| | | |
|---|---|---|
| Applicant: | Richard F Selden *et al.* | Art Unit: |
| Serial No.: | Not Yet Assigned | Examiner: |
| Filed: | Herewith | |
| Title: | *IN VIVO* PRODUCTION AND DELIVERY OF ERYTHROPOIETIN OR INSULINOTROPIN FOR GENE THERAPY | |

BOX PATENT APPLICATION
Assistant Commissioner for Patents
Washington, D.C. 20231

PRELIMINARY AMENDMENT

Prior to examination of the above-captioned patent application, which is being filed herewith, kindly amend the application as follows.

In the Specification:

On page 1, line 5, after "This application is" and before "a continuation-in-part" insert --a continuation of U.S. Serial No. 08/334,455, filed November 4, 1994, which is a continuation of U.S. Serial No. 07/911,533, filed July 10, 1992, which is--.

In the Claims:

Cancel claims 68-107.

## CONCLUSION

Although no fees are believed to be due, if there are any charges or any credits, please apply them to Deposit Account No. 03-2095.

Respectfully submitted,

Date: June 8, 1999

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Sandra E. Marxen
Printed name of person mailing correspondence

Signature of person mailing correspondence

# APPLICATION

# FOR

# UNITED STATES LETTERS PATENT

APPLICANT : Richard F Selden, Douglas Treco & Michael W. Heartlein
TITLE : *IN VIVO* PRODUCTION AND DELIVERY OF ERYTHROPOIETIN OR INSULINOTROPIN FOR GENE THERAPY

\\Ceserver\documents\50010\50010.007006 Application Cover Sheet.wpd

# IN VIVO PRODUCTION AND DELIVERY OF ERYTHROPOIETIN OR INSULINOTROPIN FOR GENE THERAPY

## Description

### Related Applications

This application is a continuation-in-part of U. S. Serial No. 07/787,840, filed November 5, 1991, entitled "In Vivo Protein Production and Delivery System for Gene Therapy" and of U. S. Serial No. 07/789,188, filed November 5, 1991, entitled "Targeted Introduction of DNA Into Primary or Secondary Cells and Their Use for Gene Therapy". The teachings of these applications are incorporated by reference.

### Background of the Invention

A variety of congenital, acquired, or induced syndromes are associated with insufficient numbers of erythrocytes (red blood cells or RBCs). The clinical consequence of such syndromes, collectively known as the anemias, is a decreased oxygen-carrying potential of the blood, resulting in fatigue, weakness, and failure-to-thrive. Erythropoietin (EPO), a glycoprotein of molecular mass 34,000 daltons, is synthesized and released into the systemic circulation in response to reduced oxygen tension in the blood. EPO, primarily synthesized in the kidney and, to a lesser extent, in the liver, acts on erythroid precursor cells [Colony Forming Units-Erythroid (CFU-E) and Burst-Forming Units-Erythroid (BFU-E)] to promote differentiation into reticulocytes and, ultimately, mature erythrocytes.

The kidney is the major site of EPO production and, thus, renal failure or nephrectomy can lead to decreased EPO synthesis, reduced RBC numbers, and, ultimately, severe anemia as observed in predialysis and dialysis patients. Subnormal RBC counts may also result from the toxic effects of chemotherapeutic agents or azidothymidine (AZT) (used in the treatment of cancers and AIDS, respectively) on erythroid precursor cells. In addition, a variety of acquired and congenital syndromes, such as aplastic anemia, myeloproliferative syndrome, malignant lymphomas, multiple myeloma, neonatal prematurity, sickle-cell anemia, porphyria cutanea tarda, and Gaucher's disease include anemia as one clinical manifestation of the syndrome.

Purified human EPO or recombinant human EPO may be administered to patients in order to alleviate anemia by increasing erythrocyte production. Typically, the protein is administered by regular intravenous injections. The administration of EPO by injection is an imperfect treatment. Normal individuals maintain a relatively constant level of EPO, which is in the range of 6-30 mU/ml, depending on the assay used. After typical treatment regimens, serum EPO levels may reach 3,000-5,000 mU/Ml following a single injection, with levels falling over time as the protein is cleared from the blood.

If a relatively constant level of EPO is to be provided in the blood (i.e., to mimic the normal physiology of the protein), a delivery system that is capable of releasing a continuous, precisely dosed quantity of EPO into the blood is necessary.

Summary of the Invention

The present invention relates to transfected primary and secondary somatic cells of vertebrate origin, particularly mammalian origin, transfected with exogenous genetic material (DNA or RNA) which encodes a clinically useful product, such as erythropoietin (EPO) or insulinotropin [e.g. derivatives of glucagon-like peptide 1 (GLP-1) such as GLP(7-37), GLP(7-36), GLP-1(7-35) and GLP-1(7-34) as well as their carboxy-terminal amidated derivatives produced by in vivo amidating enzymes and derivatives which have amino acid alterations or other alterations which result in substantially the same biological activity or stability in the blood as that of a truncated GLP-1 or enhanced biological activity or stability], methods by which primary and secondary cells are transfected to include exogenous genetic material encoding EPO or insulinotropin, methods of producing clonal cell strains or heterogenous cell strains which express exogenous genetic material encoding EPO or insulinotropin, a method of providing EPO or insulinotropin in physiologically useful quantitites to an individual in need thereof, through the use of transfected cells of the present invention or by direct injection of DNA encoding EPO into an individual; and methods of producing antibodies against the encoded product using the transfected primary or secondary cells. Transfected cells containing EPO-encoding exogenous genetic material express EPO and, thus, are useful for preventing or treating conditions in which EPO production and/or utilization are inadequate or compromised, such as in any condition or disease in which there is anemia.

Similarly, transfected cells containing insulinotropin-encoding exogenous genetic material express insulinotropin and, thus, are useful for treating individuals in whom insulin secretion, sensitivity or function is compromised (e.g., individuals with insulin-dependent or non-insulin dependent diabetes).

The present invention includes primary and secondary somatic cells, such as fibroblasts, keratinocytes, epithelial cells, endothelial cells, glial cells, neural cells, formed elements of the blood, muscle cells, other somatic cells which can be cultured and somatic cell precursors, which have been transfected with exogenous DNA encoding EPO or exogenous DNA encoding insulinotropin. The exogenous DNA is stably integrated into the cell genome or is expressed in the cells episomally. The exogenous DNA encoding EPO is introduced into cells operatively linked with additional DNA sequences sufficient for expression of EPO in transfected cells. The exogenous DNA encoding EPO is preferably DNA encoding human EPO but, in some instances, can be DNA encoding mammalian EPO of non-human origin. EPO produced by the cells is secreted from the cells and, thus, made available for preventing or treating a condition or disease (e.g., anemia) in which EPO production and/or utilization is less than normal or inadequate for maintaining a suitable level of RBCs. Cells produced by the present method can be introduced into an animal, such as a human, in need of EPO and EPO produced in the cells is secreted into the systemic circulation. As a result, EPO is made available for prevention or treatment of a condition in which EPO production and/or utilization is

less than normal or inadequate to maintain a suitable level of RBCs in the individual. Similarly, exogenous DNA encoding insulinotropin is introduced into cells operatively linked with additional DNA sequences sufficient for expression of insulinotropin in transfected cells. The encoded insulinotropin is made available to prevent or treat a condition in which insulin production or function is compromised or glucagon release from the pancreas is to be inhibited.

Primary and secondary cells transfected by the subject method can be seen to fall into three types or categories: 1) cells which do not, as obtained, produce and/or secrete the encoded protein (e.g., EPO, insulinotropin; 2) cells which produce and/or secrete the encoded protein (e.g., EPO, insulinotropin) but in lower quantities than normal (in quantities less than the physiologically normal lower level) or in defective form, and 3) cells which make the encoded protein (e.g., EPO or insulinotropin) at physiologically normal levels, but are to be augmented or enhanced in their production and/or secretion of the encoded protein.

Exogenous DNA encoding EPO is introduced into primary or secondary cells by a variety of techniques. For example, a construct which includes exogenous DNA encoding EPO and additional DNA sequences necessary for expression of EPO in recipient cells is introduced into primary or secondary cells by electroporation, microinjection, or other means (e.g., calcium phosphate precipitation, modified calcium phosphate precipitation, polybrene precipitation, microprojectile bombardment, liposome fusion, receptor-mediated DNA delivery).

Alternatively, a vector, such as a retroviral vector, which includes exogenous DNA encoding EPO can be used, and cells can be genetically modified as a result of infection with the vector. Similarly, exogenous DNA encoding insulinotropin is introduced into primary or secondary cells using one of a variety of methods.

In addition to exogenous DNA encoding EPO or insulinotropin, transfected primary and secondary cells may optionally contain DNA encoding a selectable marker, which is expressed and confers upon recipient cells a selectable phenotype, such as antibiotic resistance, resistance to a cytotoxic agent, nutritional prototrophy or expression of a surface protein. Its presence makes it possible to identify and select cells containing the exogenous DNA. A variety of selectable marker genes can be used, such as neo, gpt, dhfr, ada, pac, hyg, mdr and hisD.

Transfected cells of the present invention are useful, as populations of transfected primary cells, transfected clonal cell strains, transfected heterogenous cell strains, and as cell mixtures in which at least one representative cell of one of the three preceding categories of transfected cells is present, as a delivery system for treating an individual with a condition or disease which responds to delivery of EPO (e.g. anemia) or for preventing the development of such a condition or disease. In the method of the present invention of providing EPO, transfected primary cells, clonal cell strains, or heterogenous cell strains, are administered to an individual in need of EPO, in sufficient quantity and by an appropriate route, to deliver EPO to the

systemic circulation at a physiologically relevant level. In a similar manner, transfected cells of the present invention providing insulinotropin are useful as populations of transfected primary cells, transfected clonal cell strains, transfected heterogenous cell strains, and as cell mixtures, as a delivery system for treating an individual in whom insulin production, secretion or function is compromised or for inhibiting (totally or partially) glucagon secretion from the pancreas. A physiologically relevant level is one which either approximates the level at which the product is normally produced in the body or results in improvement of an abnormal or undesirable condition.

Clonal cell strains of transfected secondary cells (referred to as transfected clonal cell strains) expressing exogenous DNA encoding EPO (and, optionally, including a selectable marker gene) are produced by the method of the present invention. The present method includes the steps of: 1) providing a population of primary cells, obtained from the individual to whom the transfected primary cells will be administered or from another source; 2) introducing into the primary cells or into secondary cells derived from primary cells a DNA construct which includes exogenous DNA encoding EPO and additional DNA sequences necessary for expression of EPO, thus producing transfected primary or secondary cells; 3) maintaining transfected primary or secondary cells under conditions appropriate for their propagation; 4) identifying a transfected primary or secondary cell; and 5) producing a colony from the transfected primary or secondary cell identified in (4) by maintaining it under

appropriate culture conditions and for sufficient time for its propagation, thereby producing a cell strain derived from the (founder) cell identified in (4). In one embodiment of the method, exogenous DNA encoding EPO is introduced into genomic DNA by homologous recombination between DNA sequences present in the DNA construct used to transfect the recipient cells and the recipient cell's genomic DNA. Clonal cell strains of transfected secondary cells expressing exogenous DNA encoding insulinotropin (and, optionally, including a selectable marker gene) are also produced by the present method.

In one embodiment of the present method of producing a clonal population of transfected secondary cells, a cell suspension containing primary or secondary cells is combined with exogenous DNA encoding EPO and DNA encoding a selectable marker, such as the bacterial neo gene. The two DNA sequences are present on the same DNA construct or on two separate DNA constructs. The resulting combination is subjected to electroporation, generally at 250-300 volts with a capacitance of 960 $\mu$Farads and an appropriate time constant (e.g., 14 to 20 msec) for cells to take up the DNA construct. In an alternative embodiment, microinjection is used to introduce the DNA construct containing EPO-encoding DNA into primary or secondary cells. In either embodiment, introduction of the exogenous DNA results in production of transfected primary or secondary cells. Using the same approach, electroporation or microinjection is used to produce a clonal population of transfected secondary cells containing exogenous DNA encoding insulinotropin alone, or insulinotropin and a selectable marker.

In the method of producing heterogenous cell strains of the present invention, the same steps are carried out as described for production of a clonal cell strain, except that a single transfected primary or secondary cell is not isolated and used as the founder cell. Instead, two or more transfected primary or secondary cells are cultured to produce a heterogenous cell strain.

The subject invention also relates to a method of producing antibodies specific for EPO. In the method, transfected primary or secondary cells expressing EPO are introduced into an animal recipient (e.g., rabbit, mouse, pig, dog, cat, goat, guinea pig, sheep, non-human primate). The animal recipient produces antibodies against the EPO expressed, which may be the entire EPO protein antigen or a peptide encoded by a fragment of the intact EPO gene. Polyclonal sera is obtained from the animals. It is also possible to produce monoclonal antibodies through the use of transfected primary or secondary cells. Splenocytes are removed from an animal recipient of transfected primary or secondary cells expressing EPO. The splenocytes are fused with myeloma cells, using known methods, such as that of Koprowski et al. (U.S. Patent No. 4,172,124) or Kohler et al., (Nature 256:495-497 (1975)) to produce hybridoma cells which produce the desired anti-EPO monoclonal antibody. The polyclonal antisera and monoclonal antibodies produced can be used for the same purposes (e.g., diagnostic, preventive, or therapeutic purposes) as antibodies produced by other methods. Similarly, antibodies specific for insulinotropin can be produced by the method of the present invention.

The present invention is particularly advantageous in treating anemia and other conditions in which EPO production, utilization or both is compromised in that it: 1) makes it possible for one gene therapy treatment, when necessary, to last a patient's lifetime; 2) allows precise dosing (the patient's cells continuously determine and deliver the optimal dose of EPO based on physiologic demands, and the stably transfected cell strains can be characterized extensively in vitro prior to implantation, leading to accurate predictions of long term function in vivo); 3) is simple to apply in treating patients; 4) eliminates issues concerning patient compliance (periodic administration of EPO is no longer necessary); and 5) reduces treatment costs (since the therapeutic protein is synthesized by the patient's own cells, investment in costly protein production and purification facilities is unnecessary).

Brief Description of the Drawings

Figure 1 is a schematic representation of plasmid pXEPO1. The solid black arc represents the pUC12 backbone and the arrow denotes the direction of transcription of the ampicillin resistance gene. The stippled arc represents the mouse metallothionein promoter (pmMT1). The unfilled arc interrupted by black boxes represents the human erythropoietin EPO gene (the black boxes denote exons and the arrow indicates the direction hEPO transcription). The relative positions of restriction endonuclease recognition sites are indicated.

Figure 2 is a schematic representation of plasmid pcDNEO. This plasmid has the neo gene from plasmid

pSV2neo (a BamHI-BgIII fragment) inserted into the BamHI site of plasmid pcD; the amp and pBR322ori sequences are from pBR322; the polyA, 19S splice junction, and early promoter sequences are from SV40.

Figure 3 is a schematic representation of plasmid pXGH301. This plasmid contains both the human growth hormone (hGH) and neo resistance genes. Arrows indicate the directions of transcription of the various genes. The positions of restriction endonuclease recognition sites, the mouse metallothionein promoter (pMMT1), the amp resistance gene, and the SV40 early promoter (pSV40 early) are indicated.

Figure 4 is a schematic representation of plasmid pE3neoEPO. The positions of the human erythropoietin gene and the neo and amp resistance genes are indicated. Arrows indicate the directions of transcription of the various genes. pmMT1 denotes the mouse metallothionein promoter (driving hEPO expression) and pTK denotes the Herpes Simplex Virus thymidine kinase promoter (driving neo expression). The dotted regions of the map mark the positions of human HGPRT sequences. The relative positions of restriction endonuclease recognition sites are indicated.

Figure 5A shows results of Western blot analysis of hEPO secreted by normal human fibroblasts cotransfected with pXEPO1 and pcDNEO. The left panel shows the Western analysis and the right panel shows a photograph of the Coomassie blue stained gel. Lanes C,E, and M signify Control sample (supernatant from untransfected human fibroblasts), Experimental sample (supernatant from a

clonal strain of human fibroblasts expressing hEPO), and marker lanes, respectively.

Figure 5B shows results of Western blot analysis of hEPO secreted by normal human fibroblasts cotransfected with pXEPO1 and pcDNEO. Supernatant from a clonal strain of human fibroblasts expressing hEPO (lane 1) was further analyzed for glycosylation by treatment with endoglycosidase-F (lane 2), neuraminidase (lane 3), and O-glycanase (lane 4).

Fibure 6A shows results of an assay to detect hEPO in the serum of mice implanted with transfected rabbit fibroblasts expressing hEPO.

Figure 6B shows hematocrit (HCT) levels in control mice and mice implanted with transfected rabbit fibroblasts expressing hEPO.

## Detailed Description of the Invention

The present invention relates to the use of genetically engineered cells to deliver a clinically useful or otherwise desirable substance to an individual in whom production of the substance is desired (e.g., to prevent or treat a disease or condition in which the product is produced or functions at an unacceptable level). In particular, it relates to the use of genetically engineered cells to deliver EPO to the systemic circulation of an individual in need of EPO, resulting in an increase in mature red blood cell numbers, an increase in the oxygen-carrying potential of the blood, and an alleviation of the symptoms of anemia. The present invention provides a means of delivering EPO at physiologically

relevant levels and on a continuous basis to an individual. It further particularly relates to the use of genetically engineered cells to deliver insulinotropin to an individual in need of insulinotropin to stimulate insulin release, to increase insulin sensitivity in peripheral tissues, or to inhibit glucagon secretion from the pancreas.

As used herein, the term primary cell includes cells present in a suspension of cells isolated from a vertebrate tissue source (prior to their being plated, i.e., attached to a tissue culture substrate such as a dish or flask), cells present in an explant derived from tissue, both of the previous types of cells plated for the first time, and cell suspensions derived from these plated cells. The term secondary cell or cell strain refers to cells at all subsequent steps in culturing. That is, the first time a plated primary cell is removed from the culture substrate and replated (passaged), it is referred to herein as a secondary cell, as are all cells in subsequent passages. Secondary cells are cell strains which consist of secondary cells which have been passaged one or more times. A cell strain consists of secondary cells that: 1) have been passaged one or more times; 2) exhibit a finite number of mean population doublings in culture; 3) exhibit the properties of contact-inhibited, anchorage dependent growth (anchorage-dependence does not apply to cells that are propagated in suspension culture); and 4) are not immortalized. A "clonal cell strain" is defined as a cell strain that is derived from a single founder cell. A "heterogenous cell strain" is

defined as a cell strain that is derived from two or more founder cells.

As described herein, primary or secondary cells of vertebrate, particularly mammalian, origin have been transfected with exogenous DNA encoding EPO and shown to produce the encoded EPO reproducibly, both in vitro and in vivo, over extended periods of time. In addition, the transfected primary and secondary cells have been shown to express EPO in vivo at physiologically relevant levels. The EPO expressed has been shown to have the glycosylation pattern typical of EPO purified from human urine or recombinant human EPO. This demonstration is in sharp contrast to what one of skill in the art would predict, since, for example, even experts in the field see the finite life span of normal somatic cells and the inability to isolate or grow the relevant transplantable cells as precluding their use for gene therapy unless the cells are genetically modified using retroviruses (Miller, A.D., Blood, 76:271-278 (1990)). However, the transplantation of retrovirally treated fibroblasts has been shown to provide, at best, only transient metabolic improvements, and is seen to have serious limitations as a therapeutic system. In addition, until Applicants' work, this had not been done for EPO. Normal (non immortal) fibroblasts are characterized as being "much more difficult to transfect than continuous cell lines by using calcium phosphate precipitation techniques." (Miller, A.D., Blood, 76:271-278 (1990)). Furthermore, in considering non-retroviral techniques for gene therapy, it is typical of experts in the field to believe "...the efficiency of gene delivery is dismal...A

physician would have to obtain an impossible number of cells from patients to guarantee the appropriate alteration of the millions required for therapy." (Verma, I.M. Scient. Amer., November 1990, pages 68-84).

Surprisingly, Applicants have been able to produce transfected primary and secondary cells which include exogenous DNA encoding EPO and express the exogenous DNA.

The transfected primary or secondary cells may also include DNA encoding a selectable marker which confers a selectable phenotype upon them, facilitating their identification and isolation. Applicants have also developed methods for producing transfected primary or secondary cells which stably express exogenous DNA encoding EPO, clonal cell strains and heterogenous cell strains of such transfected cells, methods of producing the clonal and heterogenous cell strains, and methods of using transfected cells expressing EPO to deliver the encoded product to an individual mammal at physiologically relevant levels. The constructs and methods are useful, for example, for treating an individual (human) whose EPO production and/or function is in need of being increased or enhanced [e.g., is compromised or less than normal, or normal but the individual would benefit from enhancement, at least temporarily, of red blood cell production (e.g., during predialysis or dialysis therapy, during treatment of AIDS with AZT, after surgery, or during chemotherapy)].

As also described herein, it is possible to transfect primary or secondary cells of vertebrate, particularly mammalian, origin with exogenous DNA encoding insulinotropin and to use them to provide insulinotropin

to an individual in whom insulin production, function and/or sensitivity is compromised.

Transfected Cells

Primary and secondary cells to be transfected in order to produce EPO or insulinotropin can be obtained from a variety of tissues and include all cell types which can be maintained and propagated in culture. For example, primary and secondary cells which can be transfected by the present method include fibroblasts, keratinocytes, epithelial cells (e.g., mammary epithelial cells, intestinal epithelial cells), endothelial cells, glial cells, neural cells, formed elements of the blood (e.g., lymphocytes, bone marrow cells), muscle cells, other somatic cells which can be cultured, and precursors of these somatic cell types. Primary cells are preferably obtained from the individual to whom the transfected primary or secondary cells are administered. However, primary cells may be obtained from a donor (other than the recipient) of the same species or another species (e.g., mouse, rat, rabbit, cat, dog, pig, cow, bird, sheep, goat, horse).

Transfected primary and secondary cells can be produced, with or without phenotypic selection, as described herein, and shown to express exogenous DNA encoding EPO or exogenous DNA encoding insulinotropin.

Exogenous DNA

Exogenous DNA incorporated into primary or secondary cells by the present method is DNA encoding the desired product (e.g., EPO or insulinotropin), a functional or

active portion, or a functional equivalent of EPO or insulinotropin (a protein which has a different amino acid sequence from that of EPO but has substantially the same biological function as EPO, or a protein which has a different amino acid sequence from that of GLP-1 related peptides but functions biologically as insulinotropin). The DNA can be obtained from a source in which it occurs in nature or can be produced, using genetic engineering techniques or synthetic processes. The DNA encoding EPO or insulinotropin will generally be DNA encoding the human product (i.e., human EPO or human insulinotropin). In some cases, however, the DNA can be DNA encoding EPO or insulinotropin of non-human origin (i.e., DNA obtained from a non-human source or DNA, produced recombinantly or by synthetic methods, which encodes a non-human EPO or insulinotropin).

The DNA transfected into primary or secondary cells can encode EPO alone or EPO and another product, such as a selectable marker to facilitate selection and identification of transfected cells. Alternatively, the transfected DNA can encode insulinotropin alone or insulinotropin and another product, such as a selectable marker. After transfection into primary or secondary cells, the exogenous DNA is stably incorporated into the recipient cell's genome (along with the additional sequences present in the DNA construct used), from which it is expressed or otherwise functions. Alternatively, the exogenous DNA may exist episomally within the transfected primary or secondary cells. DNA encoding the desired product can be introduced into cells under the control of an inducible promoter, with the result that cells

produced or as introduced into an individual do not express the product but can be induced to do so (i.e., production is induced after the transfected cells are produced but before implantation or after implantation). DNA encoding the desired product can, of course, be introduced into cells in such a manner that it is expressed upon introduction (i.e., without induction).

Selectable Markers

A variety of selectable markers can be incorporated into primary or secondary cells. For example, a selectable marker which confers a selectable phenotype such as drug resistance, nutritional auxotrophy, resistance to a cytotoxic agent or expression of a surface protein, can be used. Selectable marker genes which can be used include neo, gpt, dhfr, ada, pac, hyg and hisD. The selectable phenotype conferred makes it possible to identify and isolate recipient primary or secondary cells.

DNA Constructs

DNA constructs, which include exogenous DNA encoding the desired product (e.g., EPO, insulinotropin) and, optionally, DNA encoding a selectable marker, along with additional sequences necessary for expression of the exogenous DNA in recipient primary or secondary cells, are used to transfect primary or secondary cells in which the protein (e.g., EPO, insulinotropin) is to be produced. Alternatively, infectious vectors, such as retroviral, herpes, adenovirus, adenovirus-associated,

mumps and poliovirus vectors, can be used for this purpose.

A DNA construct which includes the exogenous DNA encoding EPO and additional sequences, such as sequences necessary for expression of EPO, can be used (e.g., plasmid pXEPO1; see Figure 1). A DNA construct can include an inducible promoter which controls expression of the exogenous DNA, making inducible expression possible. Optionally, the DNA construct may include a bacterial origin of replication and bacterial antibiotic resistance markers, which allow for large-scale plasmid propagation in bacteria. A DNA construct which includes DNA encoding a selectable marker, along with additional sequences, such as a promoter, polyadenylation site, and splice junctions, can be used to confer a selectable phenotype upon transfected primary or secondary cells (e.g., plasmid pcDNEO). The two DNA constructs are co-transfected into primary or secondary cells, using methods described herein. Alternatively, one DNA construct which includes exogenous DNA encoding EPO, a selectable marker gene and additional sequences (e.g., those necessary for expression of the exogenous DNA and for expression of the selectable marker gene) can be used. Such a DNA construct (pE3neoEPO) is described in Figure 4; it includes the EPO gene and the neo gene. Similar constructs, which include exogenous DNA encoding insulinotropin and additional sequences (e.g., sequences necessary for insulinotropin expression) can be produced (e.g., plasmid pXGLP1; see Example 11). These constructs can also include DNA encoding a selectable marker, as

well as other sequences, such as a promoter, a polyadenylation site, and splice junctions.

In those instances in which DNA is injected directly into an individual, such as by injection intomusceles, the DNA construct includes the exogenous DNA and regulatory sequences necessary and sufficient for expression of the encoded product (e.g., EPO) upon entry of the DNA construct into recipient cells.

Transfection of Primary or Secondary Cells and Production of Clonal or Heterogenous Cell Strains

Transfection of cells by the present method is carried out as follows: vertebrate tissue is first obtained; this is carried out using known procedures, such as punch biopsy or other surgical methods of obtaining a tissue source of the primary cell type of interest. For example, punch biopsy is used to obtain skin as a source of fibroblasts or keratinocytes. A mixture of primary cells is obtained from the tissue, using known methods, such as enzymatic digestion or explantation. If enzymatic digestion is used, enzymes such as collagenase, hyaluronidase, dispase, pronase, trypsin, elastase and chymotrypsin can be used.

The resulting primary cell mixture can be transfected directly or it can be cultured first, removed from the culture plate and resuspended before transfection is carried out. Primary cells or secondary cells are combined with exogenous DNA encoding EPO, to be stably integrated into their genomes and, optionally, DNA encoding a selectable marker, and treated in order to accomplish transfection. The exogenous DNA and

selectable marker-encoding DNA can each be present on a separate construct (e.g., pXEPO1 and pcDNEO, see Figures 1 and 2) or on a single construct (e.g., pE3neoEPO, see Figure 4). An appropriate quantity of DNA to ensure that at least one stably transfected cell containing and appropriately expressing exogenous DNA is produced. In general, 0.1 to 500 $\mu$g DNA is used.

In one embodiment of the present method of producing transfected primary or secondary cells, transfection is effected by electroporation, as described in the Examples. Electroporation is carried out at appropriate voltage and capacitance (and corresponding time constant) to result in entry of the DNA construct(s) into the primary or secondary cells. Electroporation can be carried out over a wide range of voltages (e.g., 50 to 2000 volts) and corresponding capacitance. As described herein, electroporation is very efficient if carried out at an electroporation voltage in the range of 250-300 volts and a capacitance of 960 $\mu$Farads (see Examples 4, 5, 7 and 8). Total DNA of approximately 0.1 to 500 $\mu$g is generally used. As described in the Examples, total DNA of 60 $\mu$g and voltage of 250-300 volts with capacitance of 960 $\mu$Farads for a time constant 14-20 of msec. has been used and shown to be efficient.

In another embodiment of the present method, primary or secondary cells are transfected using microinjection. See, for instance, Examples 4 and 9. Alternatively, known methods such as calcium phosphate precipitation, modified calcium phosphate precipitation and polybrene precipitation, liposome fusion and receptor-mediated gene delivery can be used to transfect cells. A stably,

transfected cell is isolated and cultured and subcultivated, under culturing conditions and for sufficient time, to propagate the stably transfected secondary cells and produce a clonal cell strain of transfected secondary cells. Alternatively, more than one transfected cell is cultured and subculturated, resulting in production of a heterogenous cell strain.

Transfected primary or secondary cells undergo a sufficient number of doublings to produce either a clonal cell strain or a heterogenous cell strain of sufficient size to provide EPO to an individual in effective amounts. In general, for example, 0.1 $cm^2$ of skin is biopsied and assumed to contain 100,000 cells; one cell is used to produce a clonal cell strain and undergoes approximately 27 doublings to produce 100 million transfected secondary cells. If a heterogenous cell strain is to be produced from an original transfected population of approximately 100,000 cells, only 10 doublings are needed to produce 100 million transfected cells.

The number of required cells in a transfected clonal or heterogenous cell strain is variable and depends on a variety of factors, which include but are not limited to, the use of the transfected cells, the functional level of the exogenous DNA in the transfected cells, the site of implantation of the transfected cells (for example, the number of cells that can be used is limited by the anatomical site of implantation), and the age, surface area, and clinical condition of the patient. To put these factors in perspective, to deliver therapeutic levels of EPO in an otherwise healthy 60 kg patient with anemia, the number of cells needed is approximately the

volume of cells present on the very tip of the patient's thumb.

Episomal Expression of Exogenous DNA

DNA sequences that are present within the cell yet do not integrate into the genome are referred to as episomes. Recombinant episomes may be useful in at least three settings: 1) if a given cell type is incapable of stably integrating the exogenous DNA; 2) if a given cell type is adversely affected by the integration of DNA; and 3) if a given cell type is capable of improved therapeutic function with an episomal rather than integrated DNA.

Using the transfection and culturing approaches to gene therapy described in Examples 1 and 2, exogenous DNA encoding EPO, in the form of episomes can be introduced into vertebrate primary and secondary cells. Plasmid pE3neoEPO can be converted into such an episome by the addition DNA sequences for the Epstein-Barr virus origin of replication and nuclear antigen [Yates, J.L. Nature 319:780-7883 (1985)]. Alternatively, vertebrate autonomously replicating sequences can be introduced into the construct (Weidle, U.H. Gene 73(2):427-437 (1988). These and other episomally derived sequences can also be included in DNA constructs without selectable markers, such as pXEPO1. The episomal exogenous DNA is then introduced into primary or secondary vertebrate cells as described in this application (if a selective marker is included in the episome, a selective agent is used to treat the transfected cells). Similarly, episomal expression of DNA encoding insulinotropin can be

accomplished in vertebrate primary or secondary cells, using the same approach described above with reference to EPO.

Implantation of Clonal Cell Strain or Heterogenous Cell Strain of Transfected Secondary Cells

The transfected cells produced as described above are introduced into an individual to whom EPO is to be delivered, using known methods. The clonal cell strain or heterogenous cell strain is introduced into an individual, using known methods, using various routes of administration and at various sites (e.g., renal subcapsular, subcutaneous, central nervous system (including intrathecal), intravascular, intrahepatic, intrasplanchnic, intraperitoneal (including intraomental), or intramuscular implantation)]. Once implanted in the individual, the transfected cells produce EPO encoded by the exogenous DNA. For example, an individual who has been diagnosed as anemic is a candidate for a gene therapy cure. The patient has a small skin biopsy performed; this is a simple procedure which can be performed on an out-patient basis. The piece of skin, approximately 0.1 $cm^2$, is taken, for example, from under the arm and requires about one minute to remove. The sample is processed, resulting in isolation of the patient's cells (in this case, fibroblasts) and genetically engineered to produce EPO. Based on the age, weight, and clinical condition of the patient, the required number of cells is grown in large-scale culture. The entire process usually requires 4-6 weeks and, at the end of that time, the appropriate number of genetically-engineered cells is

introduced into the individual (e.g., by injecting them back under the patient's skin). The patient is now capable of producing his or her own EPO or additional EPO.

Transfected cells, produced as described above, which contain insulinotropin-encoding DNA are delivered into an individual in whom insulin production, secretion, function and/or sensitivity is compromised. They are introduced into the individual by known methods and at various sites of administration (e.g., renal, subcapsular, subcutaneous, central nervous system (including intrathecal), intravascular, intrahepatic, intrasplanchnic, intraperitoneal (including intraomental) or intramuscular implantation). Once implanted in the individual, the transfected cells produce insulinotropin encoded by the exogenous DNA. For example, an individual in whom insulin production, secretion or sensitivity is impaired can receive therapy or preventive treatment through the implantation of transfected cells expressing exogenous DNA encoding insulinotropin produced as described herein. The cells to be genetically engineered are obtained as described above for EPO, processed in a similar manner to produce sufficient numbers of cells, and introduced back into the individual.

As this example suggests, the cells used will generally be patient-specific genetically-engineered cells. It is possible, however, to obtain cells from another individual of the same species or from a different species. Use of such cells might require administration of an immunosuppressant, alteration of histocompatibility antigens, or use of a barrier device to prevent rejection of the implanted cells.

In one embodiment, a barrier device is used to prevent rejection of implanted cells obtained from a source other than the recipient (e.g., from another human or from a non-human mammal such as a cow, dog, pig, goat, sheep or rodent). In this embodiment, transfected cells of the present invention are placed within the barrier device, which is made of a material (e.g., a membrane such as Amicon XM-50) which permits the product encoded by the exogenous DNA to pass into the recipient's circulation or tissues but prevents contact between the cells and the recipient's immune system and thus prevents an immune response to (and possible rejection of) the cells by the recipient. Alternatively, DNA encoding EPO or insulinotropin can be introduced into an individual by direct injection, such as into muscle or other appropriate site. In this embodiment, the DNA construct includes exogenous DNA encoding the therapeutic product (e.g., EPO, insulinotropin) and sufficient regulatory sequences for expression of the exogenous DNA in recipient cells. After injection into the individual, the DNA construct is taken up by some of the recipient cells. The DNA can be injected alone or in a formulation which includes a physiologically compatible carrier (e.g., a physiological buffer) and, optionally, other components, such as agents which allow more efficient entry of the DNA construct into cells, stabilize the DNA or protect the DNA from degradation.

Uses of Transfected Primary and Secondary Cells and Cell Strains

Transfected primary or secondary cells or cell strains have wide applicability as a vehicle or delivery system for EPO. The transfected primary or secondary cells of the present invention can be used to administer EPO, which is presently administered by intravenous injection. When transfected primary or secondary cells are used, there is no need for extensive purification of the polypeptide before it is administered to an individual, as is generally necessary with an isolated polypeptide. In addition, transfected primary or secondary cells of the present invention produce the therapeutic product as it would normally be produced.

An advantage to the use of transfected primary or secondary cells of the present invention is that by controlling the number of cells introduced into an individual, one can control the amount of EPO. In addition, in some cases, it is possible to remove the transfected cells if there is no longer a need for the product. A further advantage of treatment by use of transfected primary or secondary cells of the present invention is that production can be regulated, such as through the administration of zinc, steroids or an agent which affects transcription of the EPO-encoding DNA.

Glucagon-like peptide 1 (GLP-1) and glucagon-like peptide 1 derivatives (GLP-1 derivatives) are additional molecules that can be delivered therapeutically using the in vivo protein production and delivery system described in the present invention. GLP-1 derivatives include truncated derivatives GLP-1(7-37), GLP-1(7-36),

GLP-1(7-35) GLP-1(7-34) and other truncated carboxy-terminal amidated derivatives and derivatives of GLP-1 which have amino acid substitutions, deletions, additions or other alterations (e.g., addition of a non-amino acid component) which result in biological activity or stability in the blood which is substantially the same as that of a truncated GLP-1 derivative or enhanced biological activity or stability in the blood (greater than that of a truncated GLP-1 derivative). As used herein, the term GLP-1 derivative includes all of the above-described molecules. The term GLP-1 related peptide, as used herein, includes GLP-1 and GLP-1 derivatives. GLP-1 derivatives, also known as insulinotropins or incretins, are normally secreted into the circulation by cells in the gastrointestinal tract. In vivo studies have demonstrated that these peptides function to stimulate insulin secretion and inhibit glucagon secretion from the endocrine pancreas, as well as increase insulin sensitivity in peripheral tissues [Goke, R. et al. (1991) Eur. J. Clin. Inv. 21:135-144; Gutniak, M. et al. (1992) New Engl. J. Med. 326:1316-1322]. Patients with non-insulin dependent diabetes mellitus (NIDDM) are often treated with high levels of insulin to compensate for their decreased insulin sensitivity. Thus, the stimulation of insulin release and the increase in insulin sensitivity by GLP-1 derivatives would be beneficial for NIDDM patients. Of particular importance is the fact that the insulinotropin-induced stimulation of insulin secretion is strongly dependent on glucose levels, suggesting that these peptides act in response to increases in blood

glucose in vivo to potentiate insulin release and, ultimately, lower blood glucose.

The present invention will now be illustrated by the following examples, which are not intended to be limiting in any way.

## EXAMPLES

### EXAMPLE 1. ISOLATION OF FIBROBLASTS

a. Source of Fibroblasts.

Human fibroblasts can be obtained from a variety of tissues, including biopsy specimens derived from liver, kidney, lung and skin. The procedures presented here are optimized for the isolation of skin fibroblasts, which are readily obtained from individuals of any age with minimal discomfort and risk (embryonic and fetal fibroblasts may be isolated using this protocol as well). Minor modifications to the protocol can be made if the isolation of fibroblasts from other tissues is desired.

Human skin is obtained following circumcision or punch biopsy. The specimen consists of three major components: the epidermal and dermal layers of the skin itself, and a fascial layer that adheres to the dermal layer. Fibroblasts can be isolated from either the dermal or fascial layers.

b. Isolation of Human Fascial Fibroblasts. Approximately 3 $cm^2$ tissue is placed into approximately 10 ml of wash solution (Hank's Balanced Salt Solution containing 100 units/ml penicillin G, 100 $\mu$g/ml streptomycin sulfate, and 0.5 $\mu$g/ml Fungisone) and subjected to gentle agitation for a total of three 10-minute washes at room

temperature. The tissue is then transferred to a 100 mm tissue culture dish containing 10 ml digestion solution (wash solution containing 0.1 units/ml collagenase A, 2.4 units/ml grade II Dispase).

Under a dissecting microscope, the skin is adjusted such that the epidermis is facing down. The fascial tissue is separated from the dermal and epidermal tissue by blunt dissection. The fascial tissue is then cut into small fragments (less than 1 $mm^2$) and incubated on a rotating platform for 30 min at 37°C. The enzyme/cell suspension is removed and saved, an additional 10 cc of digestion solution is added to the remaining fragments of tissue, and the tissue is reincubated for 30 min at 37°C. The enzyme/cell suspensions are pooled, passed through a 15-gauge needle several times, and passed through a Cellector Sieve (Sigma) fitted with a 150-mesh screen. The cell suspension is centrifuged at 1100 rpm for 15 min at room temperature. The supernatant is aspirated and the disaggregated cells resuspended in 10 ml of nutrient medium (see below). Fibroblast cultures are initiated on tissue culture treated flasks (Corning) at a density of approximately 40,000 cells/$cm^2$.

c. Isolation of Human Dermal Fibroblasts. Fascia is removed from skin biopsy or circumcision specimen as described above and the skin is cut into small fragments less than 0.5 $cm^2$. The tissue is incubated with 0.25% trypsin for 60 min at 37°C (alternatively, the tissue can be incubated in trypsin for 18 hrs at 4°C). Under the dissecting microscope, the dermis and epidermis are separated. Dermal fibroblasts are then isolated as described above for fascial fibroblasts.

d. Isolation of Rabbit Fibroblasts. The procedure is essentially as described above. Skin should be removed from areas that have been shaved and washed with a germicidial solution and surgically prepared using accepted procedures.

EXAMPLE 2. CULTURING OF FIBROBLASTS

a. Culturing of Human Fibroblasts. When confluent, the primary culture is trypsinized using standard methods and seeded at approximately 10,000 cells/$cm^2$. The cells are cultured at 37°C in humidified air containing 5% $CO_2$. Human fibroblast nutrient medium (containing DMEM, high glucose with sodium pyruvate, 10-15% calf serum, 20 mM HEPES, 20 mM L-glutamine, 50 units/ml penicillin G, and 10 μg/ml streptomycin sulfate) is changed twice weekly.

b. Culturing of Rabbit Fibroblasts. The cells are trypsinized and cultured as described for human fibroblasts. Rabbit fibroblast nutrient medium consists of a 1:1 solution of MCDB-110 (Sigma) with 20% calf serum and conditioned medium. Conditioned medium is essentially human fibroblast nutrient medium (with 15% calf serum) removed from rabbit fibroblasts grown in mass culture for 2-3 days.

EXAMPLE 3. CONSTRUCTION OF A PLASMID (pXEPO1) CONTAINING THE HUMAN ERYTHROPOIETIN GENE UNDER THE CONTROL OF THE MOUSE METALLOTHIONEIN PROMOTER

The expression plasmid pXEPO1 has the hEPO gene under the transcriptional control of the mouse metallothionein (mMT) promoter. pXEPO1 is constructed as follows: Plasmid pUC19 (ATCC #37254) is digested with

KpnI and BamHI and ligated to a 0.7 kb KpnI-BgIII fragment containing the mouse metallothionein promoter [Hamer, D.H. and Walling, M., J. Mol. Appl. Gen., 1:273-288 (1982). This fragment can also be isolated by known methods from mouse genomic DNA using PCR primers designed from analysis of mMT sequences available from Genbank; i.e. MUSMTI, MUSMTIP, MUSMTIPRM]. The resulting clone is designated pXQM2.

The hEPO gene was isolated by from a bacteriophage lambda clone containing the entire hEPO gene. This bacteriophage was isolated by screening a human Sau3A-partial genomic DNA library (Stratagene) constructed in the lambda vector LAMBDA DASH with 0.77 kb fragment of the human gene. This 0.77 kb fragment was amplified from human genomic DNA using the primers shown below in the polymerase chain reaction (PCR).

HUMAN EPO PCR PRIMERS:

Oligo hEPO-1: 5'GTTTGCTCAGCTTGGTGCTTG (Seq. ID No. 1)
(positions 2214-2234 in the Genbank HUMERPA sequence)

Oligo hEPO-2: 5'TCAAGTTGGCCCTGTGACAT (Seq. ID No. 2)
(positions 2986-2967 in the Genbank HUMERPA sequence)

The amplified fragment, encompassing exons 4 and 5 of the human EPO gene, was radiolabelled and used to screen the human genomic DNA library. Phage with a 5.4 kb HindIII-BamHI fragment containing the entire human EPO gene were assumed to contain the entire gene, based on published DNA sequence and restriction enzyme mapping

data [Lin, F-K., et al., Proc. Natl. Acad. Sci. USA, 82:7580-7584 (1985)].

A 4.8 kb BstEII-BamHI fragment (BstEII site is at position 580 in Genbank HUMERPA sequence; the BamHI site is 4.8 kb 3′ of this site, outside of the sequenced region) was isolated from the bacteriophage clone. The purified fragment is made blunt-ended by treatment with the Klenow fragment of E. coli DNA polymerase and ligated to HincII digested pXQM2, which cuts in the pUC19-derived polylinker adjacent to the 3′ side of the subcloned mMT promoter. One orientation, in which the ablated BstEII site is proximal to the mMT promoter, was identified by restriction mapping and designated pXEPO1 (Figure 1).

## EXAMPLE 4. TRANSFECTION OF PRIMARY AND SECONDARY FIBROBLASTS WITH EXOGENOUS DNA AND A SELECTABLE MARKER GENE BY ELECTROPORATION AND MICROINJECTION

To prepare cells for electroporation, exponentially growing or early stationary phase fibroblasts are trypsinized and rinsed from the plastic surface with nutrient medium. An aliquot of the cell suspension is removed for counting, and the remaining cells are subjected to centrifugation as described above. The supernatant is aspirated and the pellet is resuspended in 5 ml of electroporation buffer (20 mM HEPES pH 7.3, 137 mM NaCl, 5 mM KCl, 0.7 mM $Na_2HPO_4$, 6 mM dextrose). The cells are recentrifuged, the supernatant aspirated, and the cells resuspended in electroporation buffer containing 1 mg/ml acetylated bovine serum albumin. The final cell suspension contains approximately $3x10^6$

cells/ml. Electroporation should be performed immediately following resuspension.

Supercoiled plasmid DNA is added to a sterile cuvette with a 0.4 cm electrode gap (Bio-Rad). The final DNA concentration is generally at least 120 $\mu$g/ml. 0.5 ml of the cell suspension (containing approximately $1.5x10^6$ cells) is then added to the cuvette, and the cell suspension and DNA solutions are gently mixed. Electroporation is performed with a Gene-Pulser apparatus (Bio-Rad). Capacitance and voltage are set at 960 $\mu$F and 250-300 V, respectively. As voltage increases, cell survival decreases, but the percentage of surviving cells that stably incorporate the introduced DNA into their genome increases dramatically. Given these parameters, a pulse time of approximately 14-20 mSec should be observed.

Electroporated cells are maintained at room temperature for approximately 5 min, and the contents of the cuvette are then gently removed with a sterile transfer pipette. The cells are added directly to 10 ml of prewarmed nutrient media (as above with 15% calf serum) in a 10 cm dish and incubated as described above. The following day, the media is aspirated and replaced with 10 ml of fresh media and incubated for a further 16-24 hrs. Subculture of cells to determine cloning efficiency and to select for G418-resistant colonies is performed the following day. Cells are trypsinized, counted and plated; typically, fibroblasts are plated at $10^3$ cells/10 cm dish for the determination of cloning efficiency and at $1-2x10^4$ cells/10 cm dish for G418 selection.

Human fibroblasts are selected for G418 resistance in medium consisting of 300-400 $\mu$g/ml G418 (Geneticin, disulfate salt with a potency of approximately 50%; Gibco) in fibroblasts nutrient media (with 15% calf serum). Cloning efficiency is determined in the absence of G418. The plated cells are incubated for 12-14 days, at which time colonies are fixed with formalin, stained with crystal violet and counted (for cloning efficiency plates) or isolated using cloning cylinders (for G418 plates). Electroporation and selection of rabbit fibroblasts is performed essentially as described for human fibroblasts, with the exception of the nutrient media used. Rabbit fibroblasts are selected for G418 resistance in medium containing 1 mg/ml G418.

Fibroblasts were isolated from freshly excised human foreskins. Cultures were seeded at 50,000 cells/$cm^2$ in DMEM + 10% calf serum. When cultures became confluent fibroblasts were harvested by trypsinization and transfected by electroporation. Electroporation conditions were evaluated by transfection with the plasmid pcDNEO. A representative electroporation experiment using near optimal conditions (60 $\mu$g of plasmid pcDNEO at an electroporation voltage of 250 volts and a capacitance setting of 960 $\mu$Farads) resulted in one $G418^r$ coloney per 588 treated cells (0.17% of all cells treated), or one $G418^r$ colony per 71 clonable cells (1.4%).

When nine separate electroporation experiments at near optimal conditions (60 $\mu$g of plasmid pcDNEO at an electroporation voltage of 300 volts and a capacitance setting of 960 $\mu$Farads) were performed, an average of one

G418$^r$ colony per 1,899 treated cells (0.05%) was observed, with a range of 1/882 to 1/7,500 treated cells. This corresponds to an average of one G418$^r$ colony per 38 clonable cells (2.6%).

Low passage primary human fibroblasts were converted to hGH expressing cells by co-transfection with plasmids pcDNEO and pXGH5 [Selden, R.F. et al., Mol. Cell. Biol., 6:3173-3179 (1986)]. Typically, 60 $\mu$g of an equimolar mixture of the two plasmids were transfected at near optimal conditions (electroporation voltage of 300 volts and a capacitance setting of 960 $\mu$Farads). The results of such an experiment resulted in one G418$^r$ colony per 14,705 treated cells.

hGH expression data for these and other cells isolated under identical transfection conditions are summarized below. Ultimately, 98% of all G418$^r$ colonies could be expanded to generate mass cultures.

| | |
|---|---|
| Number of G418$^r$ Clones Analyzed | 154 |
| Number of G418$^r$/hGH Expressing Clones | 65 |
| Average hGH Expression Level | 2.3 $\mu$g hGH/$10^6$ Cells/24 hr |
| Maximum hGH Expression Level | 23.0 $\mu$g hGH/$10^6$ Cells/24 hr |

Stable transfectants also have been generated by electroporation of primary or secondary human fibroblasts with pXGH301, a DNA construct in which the neo and hGH genes are present on the same plasmid molecule (Example

3). For example, 1.5 x $10^6$ cells were electroporated with 60$\mu$g pXGH301 at 300 volts and 960 $\mu$Farads. G418 resistant colonies were isolated from transfected secondary fibroblasts at a frequency of 652 G418 resistant colonies per 1.5 x $10^6$ treated cells (1 per 2299 treated cells). Approximately 59% of these colonies express hGH.

Primary and secondary human fibroblasts can also be transfected by direct injection of DNA into cell nuclei. The ability of primary and secondary human foreskin fibroblasts to be stably transfected by this method has not been previously reported. The 8 kb HindIII fragment from plasmid RV6.9h (Zheng, H. et al., Proc. Natl. Acad. Sci. USA 88:18 8067-8071 (1991)) was purified by gel electrophoresis and passage through an anion exchange column (QIAGEN Inc.). DNA at (10 $\mu$g/ml) was injected into primary or secondary human foreskin fibroblasts using 0.1 $\mu$m outer diameter glass needles. 41 $G418^r$ clones were isolated after injection of 2,000 cells (1 in 49 starting cells).

hGH expressing clones were also generated by microinjection. Plasmid pXGH301 (Figure 3) was linearized by ScaI digestion (which cuts once within the $amp^r$ gene in the pUC12 backbone), purified by passage through an anion exchange column (QIAGEN Inc.), and injected into secondary human foreskin fibroblasts using 0.1 $\mu$m outer diameter glass needles. Several DNA concentrations were used, ranging from 2.5-20 $\mu$g pXGH301/ml. Twenty G418 resistant clones were isolated after microinjection into 2,100 cells (1 in 105 starting cells). The fraction of $G418^r$ cells, is approximately 1% of all cells treated.

Nine of 10 clones analyzed were expressing hGH, with average hGH expression being 0.6 $\mu g/10^6$ cells/24 hr for clones isolated in this experiment, and 3 clones were expanded for studying long-term expression of hGH. All 3 were expressing hGH stably, with hGH still being produced through 33, 44, and 73 mpd for the 3 strains, respectively.

EXAMPLE 5. IN VITRO hEPO PRODUCTION BY TRANSFECTED SECONDARY HUMAN AND RABBIT SKIN FIBROBLASTS

1. Human Skin Fibroblasts

Fibroblasts were isolated from freshly excised human skin fibroblasts and cultured in DMEM + 15% calf serum. Electroporation (250 volts, 960 $\mu$Farads) with 60 $\mu g$ of an equimolar mixture of pcDNEO and pXEPO1 was performed on passage 1 cells and treated cells were selected in G418-containing medium (300 $\mu g$/ml G418). Colonies were isolated and expanded using standard methods. Data derived from an analysis of fifty-six individual clones is shown in Table 1 below. Cells were maintained in G418 (300 $\mu g$/ml G418) in DMEM + 15% calf serum and subcultured at a seeding density of 10,000 cells/$cm^2$. Culture medium was changed 24 hr prior to harvesting the cells for passaging. At the time of passage, an aliquot of the culture medium was removed for hEPO assay and the cells were then harvested, counted, and reseeded. hEPO concentration in the medium was determined using a commercially available ELISA (R & D Systems). hEPO levels are expressed as mU/$10^6$ cells/24 hr., and expression levels ranged from 69 to 55,591 mU/$10^6$ cells /24 hr. 19% of all G418-resistant colonies expressed detectable levels of hEPO.

TABLE 1

hEPO EXPRESSION IN FIFTY-SIX INDEPENDENT SECONDARY HUMAN FIBROBLAST CLONES ISOLATED BY CO-TRANSFECTION WITH pcDNEO AND pXEPO1

| HEPO Expression Level ($mU/10^6$cells/24 hr) | Number of Clones |
|---|---|
| <1,000 | 10 |
| 1,000-10,000 | 28 |
| 10,000-50,000 | 17 |
| >50,000 | 1 |

Clonally derived human fibroblasts isolated by co-transfection with pcDneo and pXEPO1 were analyzed for the glycosylation state of secreted hEPO. Media was collected from hEPO producing cells and immunoprecipitated with a mouse monoclonal antibody (Genzyme Corporation) specific for human erythropoietin. The immunoprecipitated material was subject to electrophoresis on a 12.5% polyacrylamide gel and transferred to a PVDF membrane (Millipore). The membrane was probed with the same anti-hEPO monoclonal antibody used for immunoprecipitation and was subsequently treated with an HRP-conjugated sheep anti-mouse IgG antisera (Cappel), followed by luminescent detection (ECL Western blotting detection kit; Amersham) to visualize hEPO through the production of a fluorescent product.

As shown in Figure 5A, a molecule with a molecular mass of approximately 34 kd reacts with an antibody specific for human erythropoietin. This is the expected size for naturally occurring, fully glycosylated human erythropoietin.

hEPO produced by transfected human fibroblast clones was further analyzed to determine if the secreted material had both N- and O-linked glycosylation characteristic of natural human erythropoietin isolated from urine or recombinant hEPO produced by chinese hamster ovary cells. Figure 5B shows a Western blot of the untreated cell supernatant (lane 1), the supernatant treated with endoglycosidase-F [(New England Nuclear); lane 2], the supernatant treated with neuraminidase [Genzyme); (lane 3)], and the supernatant treated with O-glycanase [(Genzyme); (lane 4)]. Treatment with endoglycosidase-F results in a shift in molecular weight from 34 kd to approximately 27 kd. Treatment with neuraminidase results in a barely detectable shift in band position, while treatment with O-glycanase further shifts the size of the immunoreactive band down to approximately 18.5 kd. These results are indistinguishable from those obtained with natural human erythropoietin isolated from urine or recombinant hEPO produced by Chinese hamster ovary cells (Browne, J.K. et al., Cold Spring Harbor Symp. Quant. Biol. 51:693-702 (1986)).

2. Rabbit Fibroblasts

Fibroblasts were isolated from freshly excised rabbit skin and cultured in DMEM 10% calf serum. Electroporation (250 volts, 960 $\mu$Farads) with 60 $\mu$g of an

equimolar mixture of pcDNEO and pXEPO1 was performed and treated cells were selected in G418-containing rabbit fibroblast growth medium (1 mg/ml G418; Example 2). Colonies were isolated and expanded using standard methods, and the resulting secondary cell strains were analyzed for hEPO expression. Data derived from forty-nine independent rabbit fibroblast clones is shown in Table 2 below. Expression levels in these clones ranged from 43 to 2,900,000 mU/$10^6$ cells/24 hr., and 72% of all G418-resistant clones expressed detectable levels of hEPO.

TABLE 2

hEPO EXPRESSION IN FORTY-NINE INDEPENDENT SECONDARY RABBIT FIBROBLAST CLONES ISOLATED BY CO-TRANSFECTION WITH pcDNEO AND pEEPO

| hEPO Expression Level (mU/$10^6$ cells/24 hr) | Number of Clones |
|---|---|
| <1,000 | 1 |
| 1,000-10,000 | 3 |
| 10,000-50,000 | 7 |
| 50,000-500,000 | 19 |
| >500,000 | 19 |

EXAMPLE 6. CONSTRUCTION OF A PLASMID CONTAINING BOTH THE HUMAN EPO GENE AND THE NEOMYCIN RESISTANCE GENE

A 6.9 kb HindIII fragment extending from positions 11,960-18,869 in the HPRT sequence [Genbank entry HUMHPRTB; Edwards, A. et al., Genomics, 6:593-608 (1990)] and including exons 2 and 3 of the HPRT gene, is subcloned into the HindIII site of pUC12. The resulting clone is cleaved at the unique XhoI site in exon 3 of the HPRT gene fragment and the 1.1 kb SaII-XhoI fragment containing the neo gene from pMC1NEO (Stratagene) is inserted, disrupting the coding sequence of exon 3. One orientation, with the direction of neo transcription opposite that of HPRT transcription was chosen and designated pE3Neo. pE3neo has a unique XhoI site at the junction of HPRT sequences and the 5′ side of the neo gene. pE3neo is cut with XhoI and made blunt-ended by treatment with the Klenow fragment of E. coli DNA polymerase.

To insert the hEPO gene into the neo selection plasmid pE3Neo, a 5.1 kb EcoRI-HindIII fragment was isolated from plasmid pXEPO1 (Example 3; Figure 1). The EcoRI site is located adjacent to the 5′ side of the mMT promoter, and the HindIII site is located 5.1 kb away, 3′ to the hEPO coding region. The purified Fragment is made blunt-ended by treatment with Klenow fragment of E. coli DNA polymerase and ligated to the XhoI digested and blunt-ended pE3neo fragment described above. After transformation into E. coli, a plasmid with one copy of the mMT-hEPO fragment inserted into pE3neo was identified by restriction enzyme analysis in which the hEPO gene is transcribed in the same orientation as the adjacent neo

gene. This plasmid was designated pE3neoEPO. In addition to allowing direct selection of hEPO expressing $G418^r$ clones, this fragment may also be used in gene targeting to direct the integration of the hEPO gene to the human HPRT locus.

EXAMPLE 7. ISOLATION OF HUMAN FIBROBLAST CLONES EXPRESSING hEPO GENE AND A SELECTABLE MARKER (pE3neoEPO)

Fibroblasts were isolated from freshly excised human skin fibroblasts and cultured in DMEM + 15% calf serum. Electroporation (250 volts, 960 $\mu$Farads) with 60 $\mu$g of supercoiled pE3neoEPO was performed on passage 1 cells and treated cells were selected in G418-containing medium (300 $\mu$g/ml G418). Colonies were isolated and expanded using standard methods. Data derived from an analysis of twenty-six individual clones is shown in Table 3 below. Cells were maintained in G418 (300 $\mu$g/ml G418) in DMEM + 15% calf serum and subcultured at a seeding density of 10,000 cells/$cm^2$. Culture medium was changed 24 hr prior to harvesting the cells for passaging. At the time of passage an aliquot of the culture medium was removed for hEPO assay and the cells were then harvested, counted, and reseeded. hEPO concentration in the medium was determined using a commercially available ELISA (R and D Systems). hEPO levels are expressed as mU hEPO/$10^6$ cells/24 hr, and expression levels ranged from 240 to 961,620 mU/$10^6$ cells/24 hr. 89% of all G418-resistant clones expressed detectable levels of hEPO.

TABLE 3

hEPO EXPRESSION IN TWENTY-SIX INDEPENDENT SECONDARY HUMAN FIBROBLAST CLONES ISOLATED BY TRANSFECTION WITH pE3neo-EPO

| hEPO Expression Level ($mU/10^6$ cells/24 hr) | Number of Clones |
|---|---|
| <1,000 | 2 |
| 1,000-10,000 | 2 |
| 10,000-50,000 | 9 |
| 50,000-500,000 | 12 |
| >500,000 | 1 |

hEPO expressing human fibroblast clones are also isolated by electroporation with 60 $\mu$g of HindIII digested pE3neoEPO. hEPO expressing rabbit fibroblast clones are isolated using plasmid pE3neoEPO under identical transfection conditions, with the exception that rabbit fibroblast clones are selected in rabbit fibroblast growth medium (Example 2) containing 1 mg/ml G418.

EXAMPLE 8. ISOLATION OF TRANSFECTANTS IN THE ABSENCE OF SELECTION

The high frequency of transfection in human fibroblasts (greater than 1% stable transfectant per clonable cell; Example 4) indicates that it should be possible to isolate cell clones that have stably incorporated exogenous DNA without the use of a selective agent. Stable transfection of primary fibroblasts with the

plasmid pXEPO1 should render recipient fibroblasts capable of secreting human erythropoietin into the surrounding medium. Therefore, an ELISA for hEPO (or for any expressed protein of therapeutic interest) can be used as a simple and rapid screen for transfectants. Alternatively, it should be possible to determine the true frequency of stable integration of exogenous DNA using a screening method such as PCR which does not necessarily rely on expression of transfected DNA.

1. Primary Human Fibroblasts

Approximately 2.0 x $10^6$ human cells that were freshly dissociated from tissue are electroporated with 60 $\mu$g of pXEPO1 at 300 volts, 960 $\mu$Farads. Cells are plated immediately in a 100 mm tissue culture dish containing 10 ml of prewarmed medium and incubated at 37°C in a humidified 5% $CO_2$ atmosphere. Two days following transfection, 5 x $10^3$ cells are subcultured into a 24 well cloning plate (Bellco Glass Co.). Each well of the 24 well plate contained 16 smaller wells (384 wells/plate). Eight days after plating into the 24 large wells, media is screened for hEPO expression via ELISA. A second, confirming assay, is done in which media from wells exhibiting hEPO expression is aspirated out, replaced with fresh media and assayed for hEPO 24 hours later. Colony counts at this stage should reveal approximately 10 colonies per large well.

Individual colonies in each of the 16 small wells within one of the hEPO-positive larger wells are trypsinized and transferred to wells of a 96 well plate. Three days later each of those wells are assayed for hEPO

expression. Cells from hEPO positive cells are expanded for further study. This experiment may also be performed using secondary human foreskin fibroblasts.

2. Primary Rabbit Fibroblasts

Passage 1 rabbit skin cells were transfected with pXEPO1. The electroporation conditions were identical to the human tissue electroporation described above. 1 x $10^3$ cells are subcultured into a 384 well plate. Seven days later hGH assays are performed on media from each of the 24 large wells. Cells in each of the small wells in hEPO-positive large wells are trypsinized and transferred to wells of a 96 well plate. Three days later each of these wells are assayed for hEPO expression. Cells from hEPO positive cells are expanded for further study. This experiment may also be performed using secondary rabbit skin fibroblasts.

## EXAMPLE 9. STABLE TRANSFECTION OF PRIMARY HUMAN FIBROBLASTS BY MICROINJECTION

Direct injection of DNA into cell nuclei is another method for stably transfecting cells. The ability of primary and secondary human foreskin fibroblasts to be stably transfected by this method is described in Example 4, but has not been previously reported in the literature. The 13.1 kb HindIII fragment from plasmid pE3neoEPO is purified by gel electrophoresis and passed through an anion exchange column (QIAGEN Inc.). This fragment contains the human EPO and bacterial neo genes, flanked on both sides with human HPRT sequences. DNA at (10 $\mu$g/ml) is injected into primary or secondary human foreskin fibroblasts using 0.1 $\mu$m diameter glass needles.

$G418^r$ clones are isolated approximately 12-14 days after injection. Alternatively, the total HindIII digest of pE3neoEPO, as well as linearized or supercoiled pE3neoEPO may be injected to isolate hEPO expressing cells.

EXAMPLE 10. EXPRESSION OF BIOLOGICALLY ACTIVE HUMAN ERYTHROPOIETIN IN MICE

The mouse provides a valuable system to study implants of genetically engineered cells for their ability to deliver therapeutically useful proteins to an animal's general circulation. The relative immune-incompetence of nude mice allow xenogeneic implants to retain biologic function and may allow certain primary and secondary rabbit fibroblasts to survive in vivo for extended periods.

For implantation of cells into the subrenal capsule, mice are given intraperitoneal injection of Avertin at a dose of 0.0175 ml/g body weight. The kidney (generally the left kidney) is approached through an 8-10 mm incision made approximately 3 mm below the rib cage. The skin, abdominal musculature, peritoneum, and peri-renal fascia are retracted to expose the kidney. A small forcep is used to pull the kidney out of the abdominal cavity. A 27-gauge hypodermic needle is used to make a small opening in the renal capsule. Using a 20-gauge I.V. catheter, cells to be implanted (typically 3 million cells in a volume of 5-10 $\mu$l) are withdrawn into a 1 ml syringe and slowly ejected under the renal capsule. Care is taken to ensure that the cells are released distal to the opening in the renal capsule. The incision is closed with one staple through the musculature and the skin. Blood is collected after placing the mouse in a large

beaker containing methoxyflurane until light anesthesia is achieved. The tip of a Pasteur pipette is placed between the eye and the periorbital space to collect blood from the orbital sinus. Serum hEPO levels are determined using a commercially available kit (R and D Systems). An aliquot of blood is also drawn into EDTA coated capillary tubes (Statspin, Norwood, MA) for determination of hematocrit levels.

A clonal strain of rabbit skin fibroblasts was isolated by the methods described in Example 5. One clone, designated RF115-D4, was determined to be stably transfected with the human EPO gene and expressed approximately 786,000 mU hEPO/$10^6$ cells/24 hr. Three million cells were implanted into the subrenal capsule in each of six nude mice. Approximately 400 $\mu$l of blood was drawn on days 1 and 7 after implantation and on every other day thereafter until day 21. During this time an equal volume of saline solution was injected after bleeding to prevent hypotonic shock. Blood was drawn weekly thereafter until day 63. An identical bleeding schedule was used on ten mice that had no cells implanted. Figure 6 A shows the effect of these treatments on blood hematocrit (HCT), a commonly used indicator of red blood cell number, in implanted and control animals. In control animals, HCT drops dramatically by day 7, followed by a return to approximately normal levels by day 15. In contrast, animals receiving implants of the hEPO expressing cells showed elevated HCT levels by day 7. HCT remained elevated through day 63, reaching a peak of 64%, or 1.4 times higher than the day 1 level of 45%, on day 35 after implantation. As shown in Figure 6B,

immunoreactive hEPO was readily detectable in the blood of implanted animals (the sensitivity of the hEPO ELISA has been determined to be 2 mU/ml by the kit's manufacturer (R and D Systems) and endogenous mouse EPO shows no cross-reactivity with the antibodies used in the ELISA kit). hEPO levels in the implanted animals dropped gradually, from 29 to 9 mU/ml, from days 7 to 63 post-implantation.

This Example clearly demonstrates that normal skin fibroblasts that have been genetically engineered to express and secrete hEPO can: 1) survive in vivo to deliver hEPO to an animals systemic circulation for up to 2 months, and 2) the hEPO produced is biologically functional, serving to prevent the drop in hematocrit observed in the frequently bled control animals, and resulting in a net increase in HCT even when animals were challenged with a bleeding schedule that produces an anemic response in control animals.

EXAMPLE 11. EXPRESSION OF GLP-1(7-37) FROM SECONDARY HUMAN SKIN FIBROBLASTS STRAINS AFTER TRANSFECTION WITH A GLP-1(7-37) EXPRESSION PLASMID

The portion of GLP-1 from amino acid residues 7 to 37 [GLP-1(7-37); encoded by base pairs 7214 to 7306 in Genbank sequence HUMGLUCG2] has been demonstrated to have insulinotropin activity *in vivo*. Plasmid pXGLP1 is constructed such that the GLP-1(7-37) moiety is fused at its N-terminus to a 26-amino acid signal peptide derived from human growth hormone for efficient transport through the endoplasmic reticulum. The fusion protein is cleaved

immediately C-terminal to residue 26 prior to secretion, such that the secreted product consists solely of residues 7-37 of GLP-1. Expression of the signal peptide: GLP-1(1-37) fusion protein is controlled by the mouse metallothionein promoter.

Plasmid pXGLP1 is constructed as follows: Plasmid PXGH5 [Selden, R.F. et al., Mol. Cell. Biol. 6:3173-3179 (1986)] is digested with SmaI and ligated to a double-stranded oligonucleotide containing a BgIII site (BgIII linkers; New England Biolabs). The ligated product is digested with BgIII and EcoRI and the 0.32 kb fragment corresponding to the 3′-untranslated region of the human growth hormone gene is isolated (with a BgIII linker attached to the SmaI site lying at position 6698 in Genbank entry HUMGHCSA). The hGH fragment can also be isolated by known methods from human genomic DNA using PCR primers designed to amplify the sequence between positions 6698 to 7321 in Genbank entry HUMGHCSA. A 1.45 EcoRI-BgIII fragment containing the mouse metallothionein (mMT) promoter [Hamer, D.H. and Walling, M., J. Mol. Appl. Gen., 1:273-288 (1982)] is next isolated. The mouse metallothionein promoter may be isolated by known methods from mouse genomic DNA using PCR primers designed from analysis of mMT sequences available from Genbank (i.e. Genbank entries MUSMTI, MUSMTIP, and MUSMTIPRM). Plasmid pUC19 (ATCC #37254) is digested with EcoRI and treated with bacterial alkaline phosphatase. The treated plasmid is ligated with the hGH and mMT fragments described above. The resulting plasmid has a single copy of each the mouse metallothionein promoter and the 3′untranslated region of hGH joined at a BgIII site.

This plasmid, designated pX1 is digested with BgIII and the full-length linear product is purified by gel electrophoresis.

Oligonucleotides 11.1 and 11.2 are used to amplify a DNA sequence encoding amino acids 7-37 of GLP-1 from human genomic DNA by PCR. The amplified product (104 bp) is purified and mixed with pXGH5 and oligonucleotides 11.2, 11.3, 11.4, and 11.5 and subject to PCR. Oligonucleotides 11.3 and 11.4 are complementary and correspond to the desired junction between the hGH signal peptide and GLP-1 amino acid residue 7. The 500 base pair amplification product contains 5'-untranslated, exon 1, intron 1, and part of exon 2 sequences from hGH (nucleotides 5168 to 5562 in Genbank entry HUMGHCSA) fused to a sequence encoding GLP-1 residues 7-37 followed by a stop codon. The fragment, by design, is flanked on both ends by BamHI sites.

The fragment is cleaved with BamHI and ligated to the BgIII digest of pX1 described above. Ligation products are analysed to identify those with one copy of the hGH-GLP-1(7-37) fusion product inserted at the BgIII site separating the mMT promoter and the 3'-untranslated hGH sequence in pX1, such that GLP-1 residue 37 is distal to the mMT promoter.

OLIGONUCLEOTIDES FOR AMPLIFICATION OF hGH-GLP-1(7-37) FUSION GENE

11.1 5'CATGCTGAAG GGACCTTTAC CAGT (Seq. ID No. 3)

11.2 5'TTGGATCCTT ATCCTCGGCC TTTCACCAGC CA (Seq. ID No. 4)

BamHI

11.3 5'GGCTTCAAGA GGGCAGTGCC CATGCTGAAG GGACCTTTAC CAGT (Seq. ID No. 5)

11.4 5'ACTGGTAAAG GTCCCTTCAG CATGGGCACT GCCCTCTTGA AGCC (Seq. ID No. 6)

11.5 5'AAGGATCCCA AGGCCCAACT CCCCGAAC (Seq. ID No. 7)
BamHI

11.6 5'TTGGATCCTT ATCGGCC TTTCACCAGC CA (Seq. ID No. 8)
BamHI

Alternatively, the small sizes of the signal peptide and GLP-1 moieties needed allow complete fusion genes to be prepared synthetically. DNA encoding the signal peptides of the LDL receptor (amino acid residues 1-21), preproglucagon (amino acid residues 1-20), or human growth hormone (amino acid residues 1-26) may be synthesized by known methods and ligated in vitro to similarly synthesized DNA encoding amino acids 7-37 or 7-36 of GLP-1 (followed immediately by a stop codon). The sequences necessary to design and synthesize these molecules are readily available in Genbank entries HUMLDLR01 (human LDL receptor), HUMGLUCG2 (human GLP-1 and glucagon sequences), and HUMGHCSA (human growth hormone). The ligated product may be inserted into a suitable mammalian expression vector for use in human fibroblasts. Plasmid pMSG (Pharmacia LKB Biotechnology, Piscataway, NJ) is suitable for this purpose, having 5' and 3'untranslated sequences, a splice site, a polyA addition site, and an enhancer and promoter for use in human skin fibroblasts. Alternatively, the ligated product may be synthesized with an appropriate 5'-untranslated sequence and inserted into plasmid pX1 described above.

A second insulinotropic GLP-1 derivative, GLP-1(7-36), can be expressed by substituting oligonucleotide 11.6 for oligonucleotide 11.2 described above. All subsequent cloning operations described above for construction of pXGLP1 are followed, such that the final product is lacking the C-terminal glycine residue characteristic of GLP-1(7-37). Alternatively, this terminal glycine residue may be removed in vivo by the activity of a peptidyl-glycine alpha-amidating enzyme to produce the insulinotropin GLP-1(7-36) amide.

Plasmid pXGLP1 is co-transfected into primary human skin fibroblasts with plasmid pcDNEO exactly as described for pXEPO1 and pcDNEO in Example 5. Clones are selected in G418 containing medium, transferred to 96-well plates, and assayed for GLP-1(7-37) activity or immunoreactivity in cell supernatants. GLP-1(7-37) activity is determined by incubation of cell supernatants with rat insulinoma RINm5F cells and measuring the ability of the supernatants to induce insulin secretion from these cells using a commercially available insulin radioimmunoassay (Coat-a-Count Insulin, DPC, Los Angeles, CA). GLP-1(7-37) antigen is determined using a commercially available antisera against GLP-1 (Peninsula Laboratories, Belmont, CA). GLP-1(7-37) positive clones are expanded for implantation into nude mice as described in Example 10 and blood samples are taken to monitor serum human GLP-1(7-37) levels.

In vivo activity is monitored in fasting animals by determining the insulinogenic index after intraperitoneal injection of glucose (1 mg glucose per gram of body weight). Typically, implanted and non-implanted groups

of 32 mice are fasted overnight, and 28 are injected with glucose. After injection, the 28 mice are arbitrarily assigned to seven groups of four, and blood sampling (for serum glucose and insulin) is performed on each group at 5, 10, 20, 30, 45, 60, or 90 minutes post-injection, with the non-glucose injected group serving as a fasting control. Increases in the postinjection insulinogenic index (the ration of insulin to glucose in the blood) in animals receiving GLP-1(7-37) expressing cells over non-implanted animals provides in vivo support for the insulinotropic activity of the expressed peptide.

## Equivalents

Those skilled in the art will recognize, or be able to ascertain using not more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

## Statement Regarding Correspondence of Sequence Information (Paper Copy and Disk)

A sequence listing in computer readable form and in paper form are being filed with this application. As required by 37 C.F.R. 1.821 (f), Applicants' Attorney hereby states that the content of the Sequence Listing in paper from and of the computer readable form of the "Sequence Listing" are the same.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Selden, Richard F
Treco, Douglas
Heartlein, Michael W.

(ii) TITLE OF INVENTION: In Vivo Production and Delivery of Erythropoietin or Insulinotropin for Gene Therapy

(iii) NUMBER OF SEQUENCES: 8

(iv) CORRESPONDENCE ADDRESS:
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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: US
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
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(B) REGISTRATION NUMBER: 32,227
(C) REFERENCE/DOCKET NUMBER: TKT91-01A

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CCCATATTAC GTTTGCTCAG CTTGGTGCTT G 31

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CCCATATTAC TCAAGTTGGC CCTGTGACAT 30

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CCCATATTAC CATGCTGAAG GGACCTTTAC CAGT 34

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 42 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CCCATATTAC TTGGATCCTT ATCCTCGGCC TTTCACCAGC CA 42

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 54 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CCCATATTAC GGCTTCAAGA GGGCAGTGCC CATGCTGAAG GGACCTTTAC CAGT 54

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 54 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CCCATATTAC ACTGGTAAAG GTCCCTTCAG CATGGGCACT GCCCTCTTGA AGCC 54

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CCCATATTAC AAGGATCCCA AGGCCCAACT CCCCGAAC 38

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CCCATATTAC TTGGATCCTT ATCGGCCTTT CACCAGCCA 39

CLAIMS

1. A transfected primary or secondary cell of vertebrate origin having stably integrated into its genome:

a) exogenous DNA which encodes erythropoietin and

b) DNA sequences, sufficient for expression of the exogenous DNA in the transfected primary or secondary cell,

the primary or secondary cell capable of expressing erythropoietin.

2. The transfected primary or secondary cell of vertebrate origin of Claim 1 selected from the group consisting of: transfected fibroblasts, transfected keratinocytes, transfected epithelial cells, transfected endothelial cells, transfected glial cells, transfected neural cells, transfected formed elements of the blood, transfected muscle cells, transfected hepatocytes, and transfected precursors thereof.

3. The transfected primary or secondary cell of Claim 2 which is of mammalian origin.

4. The transfected primary or secondary cell of Claim 3 selected from the group consisting of: transfected primary human cells, transfected secondary human

cells, transfected primary rabbit cells and transfected secondary rabbit cells.

5. The transfected primary or secondary cell of vertebrate origin of Claim 1 which additionally includes DNA encoding a selectable marker.

6. The transfected primary or secondary cell of Claim 5 selected from the group consisting of: transfected fibroblasts, transfected keratinocytes, transfected epithelial cells, transfected endothelial cells, transfected glial cells, transfected neural cells, transfected formed elements of the blood, transfected muscle cells, transfected hepatocytes, and transfected precursors thereof.

7. The transfected primary or secondary cell of Claim 6 which is of mammalian origin.

8. The transfected primary or secondary cell of Claim 7 selected from the group consisting of: transfected primary human cells, transfected secondary human cells, transfected primary rabbit cells, and transfected secondary rabbit cells.

9. The transfected primary or secondary cell of vertebrate origin of Claim 1 selected from the group consisting of:

a) transfected primary or secondary cells which, in their untransfected state, do not make or contain erythropoietin;

b) transfected primary or secondary cells which, in their untransfected state, make or contain erythropoietin in abnormally low amounts or in defective form; and

c) transfected primary or secondary cells which, in their untransfected form, make or contain erythropoietin in physiologically normal amounts.

10. A primary or secondary cell of vertebrate origin transfected with:

a) exogenous DNA which encodes erythropoietin; and

b) DNA sequences, sufficient for expression of the exogenous DNA in the primary or secondary cell, the sequences of (a) and (b) present in the cell in an episome.

11. The primary or secondary cell of vertebrate origin of Claim 10 selected from the group consisting of: fibroblasts, keratinocytes, epithelial cells, endothelial cells, glial cells, neural cells, formed elements of the blood, muscle cells, hepatocytes, and precursors thereof.

12. The primary or secondary cell of Claim 11 which is of mammalian origin.

13. The primary or secondary cell of Claim 12 selected from the group consisting of: primary human cells, secondary human cells, primary rabbit cells, and secondary rabbit cells.

14. A clonal cell strain of transfected secondary cells of vertebrate origin which express exogenous DNA encoding erythropoietin incorporated therein.

15. The clonal cell strain of Claim 14 wherein the exogenous DNA is stably incorporated into genomic DNA of the transfected secondary cells.

16. The clonal cell strain of Claim 15 wherein the transfected secondary cells are selected from the group consisting of: transfected secondary fibroblasts, transfected secondary keratinocytes, transfected epithelial cells, transfected endothelial cells, transfected glial cells, transfected neural cells, transfected formed elements of the blood, transfected muscle cells, transfected hepatocytes, and transfected precursors thereof.

17. The clonal cell strain of transfected secondary cells of Claim 16 wherein the transfected secondary cells are of mammalian origin.

18. The clonal strain of transfected secondary cells of Claim 17 wherein the transfected secondary cells of mammalian origin are selected from the group consisting of: transfected secondary human cells and transfected secondary rabbit cells.

19. The clonal cell strain of Claim 14 wherein the exogenous DNA is present in the transfected secondary cells in an episome.

20. A heterogenous cell strain of transfected secondary cells of vertebrate origin having stably incorporated into their genomes:

a) exogenous DNA encoding erythropoietin and

b) DNA sequences sufficient for expression of the exogenous DNA in the transfected primary or secondary cell.

the heterogenous cell strain capable of expressing erythropoietin.

21. The heterogenous cell strain of Claim 20, wherein the transfected primary or secondary cells are selected from the group consisting of: transfected fibroblasts, transfected keratinocytes, transfected epithelial cells, transfected endothelial cells, transfected glial cells, transfected neural cells, transfected formed elements of the blood, transfected muscle cells, transfected hepatocytes, and transfected precursors thereof.

22. The heterogenous cell strain of Claim 21 which is of mammalian origin.

23. A heterogenous cell strain of Claim 22 selected from the group consisting of: transfected primary human cells, transfected secondary human cells, transfected primary rabbit cells, and transfected secondary rabbit cells.

24. A mixture of cells consisting essentially of transfected primary or secondary cells of Claim 1 and untransfected primary or secondary cells.

25. A mixture of cells consisting essentially of transfected primary or secondary cells of Claim 3 and untransfected primary or secondary cells.

26. A method of producing a clonal cell strain of transfected secondary cells of vertebrate origin which express exogenous DNA encoding erythropoietin incorporated therein, comprising the steps of:

a) producing a mixture of cells of vertebrate origin containing primary cells;

b) transfecting primary cells produced in (a) with a DNA construct comprising exogenous DNA encoding erythropoietin and additional DNA sequences sufficient for expression of the exogenous DNA in the primary cells, thereby producing transfected primary cells which express the exogenous DNA encoding erythropoietin;

c) culturing a transfected primary cell which expresses the exogenous DNA encoding erythropoietin produced in (b), under conditions appropriate for propagating the transfected primary cell which expresses the exogenous DNA encoding erythropoietin, thereby producing a clonal cell strain of transfected secondary cells from the transfected primary cell.

27. The method of Claim 26 wherein the primary cells are selected from the group consisting of: fibroblasts, keratinocytes, epithelial cells, endothelial cells, glial cells, neural cells, formed elements of the blood, muscle cells, hepatocytes, and precursors thereof.

28. The method of Claim 27 wherein the transfected primary cell is of mammalian origin.

29. The method of Claim 28 wherein the primary cell is selected from the group consisting of: primary human cells, and primary rabbit cells.

30. The method of Claim 26 wherein in step (b) the primary cell of vertebrate origin is additionally transfected with DNA encoding a selectable marker.

31. The method of Claim 30 wherein the primary cell is selected from the group consisting of: fibroblasts, keratinocytes, epithelial cells, endothelial cells, glial cells, neural cells, formed elements of the blood, transfected muscle cells, hepatocytes, and precursors thereof.

32. The method of Claim 31 wherein the primary cell is of mammalian origin.

33. The method of Claim 32 wherein the primary cell is selected from the group consisting of: primary human cells and primary rabbit cells.

34. A method of producing a clonal cell strain of transfected secondary cells of vertebrate origin which express exogenous DNA encoding erythropoietin incorporated therein, comprising the steps of:

a) providing a mixture of cells of vertebrate origin containing primary cells;

b) producing a population of secondary cells from the primary cells provided in (a);

c) transfecting secondary cells produced in (b) with a DNA construct comprising exogenous DNA encoding erythropoietin and additional DNA sequences sufficient for expression of the exogenous DNA in the secondary cells, thereby producing transfected secondary cells which express the exogenous DNA encoding erythropoietin; and

d) culturing a transfected secondary cell which expresses the DNA encoding erythropoietin, produced in (c), under conditions appropriate for propagating the transfected secondary cell which expresses the exogenous DNA encoding erythropoietin,

thereby producing a clonal cell strain of transfected secondary cells from the transfected secondary cell of (d).

35. The method of Claim 34 wherein the primary cells are selected from the group consisting of: fibroblasts, keratinocytes, epithelial cells, endothelial cells, glial cells, neural cells, formed elements of the

blood, muscle cells, hepatocytes, and precursors thereof.

36. The method of Claim 35 wherein the transfected primary cell is of mammalian origin.

37. The method of Claim 36 wherein the primary cell is selected from the group consisting of: primary human cells and primary rabbit cells.

38. The method of Claim 34 wherein in step (c) the secondary cells of vertebrate origin are additionally transfected with DNA encoding a selectable marker.

39. The method of Claim 38 wherein the primary cell is selected from the group consisting of: fibroblasts, keratinocytes, epithelial cells, endothelial cells, glial cells, neural cells, formed elements of the blood, transfected muscle cells, hepatocytes, and precursors thereof.

40. The method of Claim 39 wherein the primary cell is of mammalian origin.

41. The method of Claim 40 wherein the primary cell is selected from the group consisting of: primary human cells and primary rabbit cells.

42. The method of Claim 34 wherein, in step (c), secondary cells are transfected with the DNA

construct comprising exogenous DNA encoding erythropoietin by combining the primary cells and the DNA construct comprising exogenous DNA encoding erythropoietin and subjecting the resulting combination to electroporation under conditions which result in production of at least one primary cell having exogenous DNA stably integrated into genomic DNA.

43. The method of Claim 42 wherein electroporation is carried out at an electroporation voltage of between 250 and 300 volts and a capacitance setting of approximately 960 $\mu$Farads.

44. The method of Claim 34 wherein in step (c) secondary cells are transfected with the DNA construct comprising exogenous DNA by microinjecting the DNA construct comprising exogenous DNA into the secondary cells.

45. The method of Claim 34 wherein in step (c), secondary cells are transfected with the DNA construct comprising exogenous DNA by calcium phosphate precipitation, modified calcium phosphate precipitation, liposome fusion methodologies, receptor mediated transfer, micro-projectile bombardment, and polybrene precipitation.

46. The method of Claim 34 wherein in step (c), the exogenous DNA is introduced into genomic DNA by homologous recombination between DNA sequences

present in the exogenous DNA construct and genomic DNA.

47. The method of Claim 34 additionally comprising transfecting in step (c), second cells produced in step (a) with a DNA construct comprising DNA encoding a selectable marker.

48. A method of producing a heterogenous cell strain of transfected secondary cells of vertebrate origin which express exogenous DNA encoding erythropoietin stably incorporated into the genome of said secondary cells, comprising the steps of:

a) producing a mixture of cells of vertebrate origin containing primary cells;

b) transfecting primary cells produced in (a) with exogenous DNA encoding erythropoietin and operatively linked to DNA sequences of non-retroviral origin sufficient for expression of the exogenous DNA in transfected secondary cells, thereby producing a mixture of primary cells which includes transfected primary cells which express the exogenous DNA encoding erythropoietin;

c) culturing the product of (b) under conditions appropriate for propagation of transfected primary cells which express the exogenous DNA encoding erythropoietin,

thereby producing a heterogenous cell strain of transfected secondary cells of vertebrate origin

which express the exogenous DNA encoding erythropoietin.

49. The method of Claim 48 wherein the vertebrate is a mammal and and the primary cells are selected from the group consisting of: fibroblasts, keratinocytes, epithelial cells, endothelial cells, glial cells, neural cells, formed elements of the blood, muscle cells, hepatocytes, and precursors thereof.

50. The method of Claim 48 wherein, in step (b), primary cells are transfected with the DNA construct comprising exogenous DNA encoding a therapeutic product by combining the primary cells and the DNA construct comprising exogenous DNA encoding a therapeutic product and subjecting the resulting combination to electroporation under conditions which result in production of at least one secondary cell having exogenous DNA stably integrated into genomic DNA.

51. The method of Claim 50 wherein electroporation is carried out at an electroporation voltage of between 250 and 300 volts and a capacitance setting of approximately 960 $\mu$Farads.

52. The method of Claim 48 wherein in step (b) primary cells are transfected with the DNA construct comprising exogenous DNA by microinjecting the DNA construct comprising exogenous DNA into the primary cells.

53. The method of Claim 48 wherein in step (b), secondary cells are transfected with the DNA construct comprising exogenous DNA by a method selected from the group consisting of: calcium phosphate precipitation, modified calcium phosphate precipitation, liposome fusion methodologies, receptor mediated transfer, micro-projectile bombardment, and polybrene precipitation.

54. The method of Claim 48 wherein in step (b) transfected primary cells are produced by introducing into primary cells produced in (a) a construct which undergoes homologous recombination with genomic DNA of the primary cells, thereby resulting in introduction of the construct into the genomic DNA.

55. The method of Claim 48 additionally comprising transfecting in step (b), primary cells produced in step (a) with a DNA construct comprising DNA encoding a selectable marker.

56. A method of producing a heterogenous cell strain of transfected secondary cells of vertebrate origin which express exogenous DNA encoding erythropoietin stably incorporated into the genome of said secondary cells, comprising the steps of

a) providing a mixture of cells of vertebrate origin containing primary cells;

b) producing a population of secondary cells from the primary cells provided in (a);

c) transfecting secondary cells produced in (b) with exogenous DNA encoding erythropoietin and operatively linked to DNA sequences of non-retroviral origin sufficient for expression of the exogenous DNA in transfected secondary cells, thereby producing a mixture of secondary cells which includes transfected secondary cells which express the exogenous DNA encoding erythropoietin;

d) culturing the product of (c) under conditions appropriate for propagation of transfected secondary cells which express the exogenous DNA encoding a therapeutic product,

thereby producing a heterogenous cell strain of transfected secondary cells of vertebrate origin which express the exogenous DNA encoding erythropoietin.

57. The method of Claim 56 wherein the vertebrate is a mammal and and the primary cells are selected from the group consisting of: fibroblasts, keratinocytes, epithelial cells, endothelial cells, glial cells, neural cells, formed elements of the blood, muscle cells, hepatocytes and precursors thereof.

58. The method of Claim 56 wherein, in step (c), secondary cells are transfected with the DNA construct comprising exogenous DNA encoding a therapeutic product by combining the primary cells and the DNA construct comprising exogenous DNA encoding a therapeutic product and subjecting the resulting

combination to electroporation under conditions which result in production of at least one secondary cell having exogenous DNA stably integrated into genomic DNA.

59. The method of Claim 58 wherein electroporation is carried out at an electroporation voltage of between 250 and 300 volts and a capacitance setting of approximately 960 $\mu$Farads.

60. The method of Claim 56 wherein in step (c) secondary cells are transfected with the DNA construct comprising exogenous DNA by microinjecting the DNA construct comprising exogenous DNA into the secondary cells.

61. The method of Claim 56 wherein in step (c), secondary cells are transfected with the DNA construct comprising exogenous DNA by a method selected from the group consisting of: calcium phosphate precipitation, modified calcium phosphate precipitation, liposome fusion methodologies, receptor mediated transfer, micro-projectile bombardment, and polybrene precipitation.

62. The method of Claim 58, wherein in step (c) transfected secondary cells are produced by introducing into secondary cells produced in (b) a construct which undergoes homologous recombination with genomic DNA of the secondary cells, thereby

resulting in introduction of the construct into the genomic DNA.

63. The method of Claim 60, wherein in step (c) transfected secondary cells are produced by introducing into secondary cells produced in (b) a construct which undergoes homologous recombination with genomic DNA of the secondary cells, thereby resulting in introduction of the construct into the genomic DNA.

64. The method of Claim 56 additionally comprising transfecting in step (c), secondary cells produced in step (b) with a DNA construct comprising DNA encoding a selectable marker.

65. A method of producing a clonal cell strain of secondary fibroblasts of mammalian origin which express exogenous DNA encoding erythropoietin upon introduction into a mammal, comprising the steps of:

a) providing primary fibroblasts of mammalian origin;

b) producing a population of secondary fibroblasts from the primary fibroblasts provided in (a);

c) combining the secondary fibroblasts of mammalian origin with a DNA construct comprising:

i) exogenous DNA encoding erythropoietin to be expressed in the fibroblasts; and

ii) additional DNA sequences of non-retroviral origin sufficient for expression of the exogenous DNA in the fibroblasts;

d) subjecting the combination produced in (c) to electroporation under conditions which result in transfection of the vector into the secondary fibroblasts of mammalian origin, thereby producing a mixture of transfected secondary fibroblasts of mammalian origin and non-transfected secondary fibroblasts of mammalian origin;

e) isolating a transfected secondary fibroblast of mammalian origin produced in (d); and

f) culturing the transfected secondary fibroblast of mammalian origin isolated in (e) under conditions appropriate for production of a clonal population consisting essentially of transfected secondary fibroblasts of mammalian origin which express the exogenous DNA encoding erythropoietin.

66. The method of Claim 65 wherein in step (d) electroporation is carried out at an electroporation voltage of between 250 and 300 volts and a capacitance setting of approximately 960 $\mu$Farads.

67. The method of Claim 65 further comprising maintaining the product of (f) for sufficient time and under appropriate conditions for at least 20 doublings of the transfected secondary cells which express the exogenous DNA to occur.

68. A method of providing erythropoietin in an effective amount to a mammal, comprising the steps of:

a) obtaining a source of primary cells from the mammal;

b) transfecting primary cells obtained in (a) with a DNA construct comprising exogenous DNA encoding erythropoietin and additional DNA sequences sufficient for expression of the exogenous DNA in the primary cells, thereby producing transfected primary cells which express the exogenous DNA encoding the therapeutic product;

c) culturing a transfected primary cell which expresses the exogenous DNA encoding erythropoietin produced in (b), under conditions appropriate for propagating the transfected primary cell which expresses the exogenous DNA encoding erythropoietin, thereby producing a clonal cell strain of transfected secondary cells from the transfected primary cell;

d) culturing the clonal cell strain of transfected secondary cells produced in (c) under conditions appropriate for and sufficient time for the clonal cell strain of transfected secondary cells to undergo a sufficient number of doublings to provide a sufficient number of transfected secondary cells to produce an effective amount of erythropoietin; and

e) introducing transfected secondary cells produced in (d) into the mammal in sufficient number to produce an effective amount of erythropoietin in the mammal.

69. The method of providing erythropoietin in an effective amount to a mammal of Claim 68 wherein the primary cells are selected from the group consisting of: fibroblasts, keratinocytes, epithelial cells, endothelial cells, glial cells, neural cells, formed elements of the blood, hepatocytes, and precursors thereof.

70. A method of providing erythropoietin in an effective amount to a mammal, comprising the steps of:

a) obtaining a source of primary cells from the mammal;

b) producing a population of secondary cells from the primary cells provided in (a);

c) transfecting secondary cells produced in (b) with a DNA construct comprising exogenous DNA encoding erythropoietin and additional DNA sequences sufficient for expression of the exogenous DNA in the primary cells, thereby producing transfected secondary cells which express the exogenous DNA encoding erythropoietin;

d) culturing a transfected secondary cell which expresses the exogenous DNA encoding erythropoietin produced in (c), under conditions appropriate for propagating the transfected secondary cell which expresses the exogenous DNA encoding erythropoietin, thereby producing a clonal cell strain of transfected secondary cells from the transfected secondary cell;

e) culturing the clonal cell strain of transfected secondary cells produced in (d) under conditions appropriate for and sufficient time for the clonal cell strain of transfected secondary cells to undergo a sufficient number of doublings to provide a sufficient number of transfected secondary cells to produce an effective amount of erythropoietin; and

f) introducing transfected secondary cells produced in (e) into the mammal in sufficient number to produce an effective amount of erythropoietin.

71. The method of providing a therapeutic product in an effective amount to a mammal of Claim 70 wherein the primary cells are selected from the group consisting of: fibroblasts, keratinocytes, epithelial cells, endothelial cells, glial cells, neural cells, formed elements of the blood, hepatocytes and precursors thereof.

72. A method of producing erythropoietin in an effective amount to a mammal, comprising the steps of:

a) obtaining a source of primary cells from the mammal;

b) transfecting primary cells obtained in (a) with exogenous DNA encoding erythropoietin and operatively linked to DNA sequences of non-retroviral origin sufficient for expression of the exogenous DNA in transfected secondary cells, thereby producing a mixture of primary

cells which includes transfected primary cells which express the exogenous DNA encoding erythropoietin;

c) culturing the product of (b) under conditions appropriate for propagation of transfected primary cells which express the exogenous DNA encoding erythropoietin, thereby producing a heterogenous cell strain of transfected secondary cells of vertebrate origin which express the exogenous DNA encoding erythropoietin; and

d) introducing transfected secondary cells produced in (c) into the mammal in sufficient number to produce an effective amount of erythropoietin in the mammal.

73. The method of Claim 72, wherein the primary cells are selected from the group consisting of: fibroblasts, keratinocytes, epithelial cells, endothelial cells, glial cells, neural cells, formed elements of the blood, hepatocytes, and precursors thereof.

74. A method of providing erythropoietin in an effective amount to a mammal, comprising the steps of:

a) obtaining a source of primary cells from the mammal;

b) producing a population of secondary cells from the primary cells provided in (a);

c) transfecting secondary cells produced in (b) with exogenous DNA encoding erythropoietin and operatively linked to DNA sequences of non-retroviral origin sufficient for expression of

the exogenous DNA in transfected secondary cells, thereby producing a mixture of secondary cells which includes transfected secondary cells which express the exogenous DNA encoding erythropoietin;

d) culturing the product of (c) under conditions appropriate for propagation of transfected secondary cells which express the exogenous DNA encoding erythropoietin, thereby producing a heterogenous cell strain of transfected secondary cells of vertebrate origin which express the exogenous DNA encoding erythropoietin; and

e) introducing transfected secondary cells produced in (c) into the mammal in sufficient number to produce an effective amount of erythropoietin in the mammal.

75. The method of Claim 74 wherein the primary cells are selected from the group consisting of: fibroblasts, keratinocytes, epithelial cells, endothelial cells, glial cells, neural cells, formed elements of the blood, hepatocytes, and precursors thereof.

76. A method of providing erythropoietin to a mammal at biologically significant levels, comprising administering to the mammal transfected primary or secondary cells of mammalian origin which express erythropoietin in sufficient quantity to produce physiologically relevant levels in the mammal.

77. The method of Claim 76 wherein the transfected primary or secondary cells are selected from the group consisting of primary human cells, primary rabbit cells.

78. A transfected primary or secondary cell of vertebrate origin having stably integrated into its genome:

a) exogenous DNA which encodes a glucagon-like peptide 1 related peptide with insulinotropin activity, and

b) DNA sequences, sufficient for expression of the exogenous DNA in the transfected primary or secondary cell,

the primary or secondary cell capable of expressing the glucagon-like peptide 1 related peptide.

79. The transfected primary or secondary cell of vertebrate origin of Claim 78 selected from the group consisting of: transfected fibroblasts, transfected keratinocytes, transfected epithelial cells, transfected endothelial cells, transfected glial cells, transfected neural cells, transfected formed elements of the blood, transfected muscle cells, transfected hepatocytes, and transfected precursors thereof.

80. The transfected primary or secondary cell of Claim 79 which is of mammalian origin.

81. The transfected primary or secondary cell of Claim 80 selected from the group consisting of: transfected primary human cells, transfected secondary human cells, transfected primary rabbit cells and transfected secondary rabbit cells.

82. The transfected primary or secondary cell of vertebrate origin of Claim 78 which additionally includes DNA encoding a selectable marker.

83. The transfected primary or secondary cell of Claim 82 selected from the group consisting of: transfected fibroblasts, transfected keratinocytes, transfected epithelial cells, transfected endothelial cells, transfected glial cells, transfected neural cells, transfected formed elements of the blood, transfected muscle cells, transfected hepatocytes and transfected precursors thereof.

84. The transfected primary or secondary cell of Claim 83 which is of mammalian origin.

85. The transfected primary or secondary cell of vertebrate origin of Claim 78 selected from the group consisting of:

a) transfected primary or secondary cells which, in their untransfected state, do not make or contain a glucagon-like peptide 1 related peptide;

b) transfected primary or secondary cells which, in their untransfected state, make or contain a

glucagon-like peptide 1 related peptide in abnormally low amounts or in defective form; and

c) transfected primary or secondary cells which, in their untransfected form, make or contain a glucagon-like peptide 1 related peptide in physiologically normal amounts.

86. A primary or secondary cell of vertebrate origin transfected with:

a) exogenous DNA which encodes a glucagon-like peptide 1 related peptide with insulinotropin activity; and

b) DNA sequences, sufficient for expression of the exogenous DNA in the primary or secondary cell, the sequences of (a) and (b) present in the cell in an episome.

87. A clonal cell strain of transfected secondary cells of vertebrate origin which express exogenous DNA encoding a glucagon-like peptide 1 related peptide incorporated therein.

88. The clonal cell strain of Claim 87 wherein the exogenous DNA is stably incorporated into genomic DNA of the transfected secondary cells.

89. A heterogenous cell strain of transfected secondary cells of vertebrate origin having stably incorporated into their genomes:

a) exogenous DNA encoding a glucagon-like peptide 1 related peptide with insulinotropin activity and

b) DNA sequences sufficient for expression of the exogenous DNA in the transfected primary or secondary cell,

the heterogenous cell strain capable of expressing a glucagon-like peptide 1 related peptide.

90. A mixture of cells consisting essentially of transfected primary or secondary cells of Claim 78 and untransfected primary or secondary cells.

91. A method of producing a clonal cell strain of transfected secondary cells of vertebrate origin which express exogenous DNA encoding a glucagon-like peptide 1 related peptide incorporated therein, comprising the steps of:

a) producing a mixture of cells of vertebrate origin containing primary cells;

b) transfecting primary cells produced in (a) with a DNA construct comprising exogenous DNA encoding a glucagon-like peptide 1 related peptide and additional DNA sequences sufficient for expression of the exogenous DNA in the primary cells, thereby producing transfected primary cells which express the exogenous DNA encoding a glucagon-like peptide 1 related peptide; and

c) culturing a transfected primary cell which expresses the exogenous DNA encoding a glucagon-like peptide 1 related peptide produced in (b), under conditions appropriate for propagating the transfected primary cell which expresses the exogenous DNA encoding a glucagon-like peptide 1 related peptide,

thereby producing a clonal cell strain of transfected secondary cells from the transfected primary cell.

92. A method of producing a clonal cell strain of transfected secondary cells of vertebrate origin which express exogenous DNA encoding a glucagon-like peptide 1 related peptide incorporated therein, comprising the steps of:

a) providing a mixture of cells of vertebrate origin containing primary cells;

b) producing a population of secondary cells from the primary cells provided in (a);

c) transfecting secondary cells produced in (b) with a DNA construct comprising exogenous DNA encoding a glucagon-like peptide 1 related peptide and additional DNA sequences sufficient for expression of the exogenous DNA in the secondary cells, thereby producing transfected secondary cells which express the exogenous DNA encoding a glucagon-like peptide 1 related peptide; and

d) culturing a transfected secondary cell which expresses the DNA encoding a glucagon-like peptide 1 related peptide produced in (c), under conditions appropriate for propagating the transfected secondary cell which expresses the exogenous DNA encoding a glucagon-like peptide 1 related peptide,

thereby producing a clonal cell strain of transfected secondary cells from the transfected secondary cell of (d).

93. A method of producing a heterogenous cell strain of transfected secondary cells of vertebrate origin which express exogenous DNA encoding a glucagon-like peptide 1 related peptide stably incorporated into the genome of said secondary cells, comprising the steps of:

a) producing a mixture of cells of vertebrate origin containing primary cells;

b) transfecting primary cells produced in (a) with exogenous DNA encoding a glucagon-like peptide 1 related peptide and operatively linked to DNA sequences of non-retroviral origin sufficient for expression of the exogenous DNA in transfected secondary cells, thereby producing a mixture of primary cells which includes transfected primary cells which express the exogenous DNA encoding a glucagon-like peptide 1 related peptide;

c) culturing the product of (b) under conditions

appropriate for propagation of transfected primary cells which express the exogenous DNA encoding a glucagon-like peptide 1 related peptide,

thereby producing a heterogenous cell strain of transfected secondary cells of vertebrate origin which express the exogenous DNA encoding a glucagon-like peptide 1 related peptide.

94. A method of producing a clonal cell strain of secondary fibroblasts of mammalian origin which express exogenous DNA encoding a glucagon-like peptide 1 related peptide upon incorporation into the genome of the secondary fibroblast, comprising the steps of:

a) providing primary fibroblasts of mammalian origin;

b) producing a population of secondary fibroblasts from the primary fibroblasts provided in (a);

c) combining the secondary fibroblasts of mammalian origin with a DNA construct comprising:

i) exogenous DNA encoding a glucagon-like peptide 1 related peptide to be expressed in the fibroblasts; and

ii) additional DNA sequences of non-retroviral origin sufficient for expression of the exogenous DNA in the fibroblasts;

d) subjecting the combination produced in (c) to electroporation under conditions which result in transfection of the vector into the secondary fibroblasts of mammalian origin, thereby

producing a mixture of transfected secondary fibroblasts of mammalian origin and non-transfected secondary fibroblasts of mammalian origin;

e) isolating a transfected secondary fibroblast of mammalian origin produced in (d); and

f) culturing the transfected secondary fibroblast of mammalian origin isolated in (e) under conditions appropriate for production of a clonal population consisting essentially of transfected secondary fibroblasts of mammalian origin which express the exogenous DNA encoding a glucagon-like peptide 1 related peptide.

95. A method of Claim 94 wherein the glucagon-like peptide 1 related peptide is a glucan-like peptide 1 derivative selected from the group consisting of GLP-1(7-37), GLP-1(7-36), GLP-1(7-35) GLP-1(7-34) and other truncated carboxy-terminal amidated derivatives and derivatives of GLP-1 which have amino acid substitutions, deletions, additions or other alterations (e.g., addition of a non-amino acid component) which result in biological activity or stability in the blood which is substantially the same as that of a truncated GLP-1 derivative or enhanced biological activity or stability in the blood.

96. A method of providing a glucagon-like peptide 1 related peptide in an effective amount to a mammal, comprising the steps of:

a) obtaining a source of primary cells from the mammal;

b) transfecting primary cells obtained in (a) with a DNA construct comprising exogenous DNA encoding a glucagon-like peptide 1 related peptide and additional DNA sequences sufficient for expression of the exogenous DNA in the primary cells, thereby producing transfected primary cells which express the exogenous DNA encoding a glucagon-like peptide 1 related peptide;

c) culturing a transfected primary cell which expresses the exogenous DNA encoding a glucagon-like peptide 1 related peptide produced in (b), under conditions appropriate for propagating the transfected primary cell which expresses the exogenous DNA encoding a glucagon-like peptide 1 related peptide, thereby producing a clonal cell strain of transfected secondary cells from the transfected primary cell;

d) culturing the clonal cell strain of transfected secondary cells produced in (c) under conditions appropriate for and sufficient time for the clonal cell strain of transfected secondary cells to undergo a sufficient number of doublings to provide a sufficient number of transfected secondary cells to produce an effective amount of a glucagon-like peptide 1 related peptide; and

e) introducing transfected secondary cells produced in (d) into the mammal in sufficient number to produce an effective amount of a glucagon-like peptide 1 related peptide in the mammal.

97. The method of providing a therapeutic product in an effective amount to a mammal of Claim 95 wherein the primary cells are selected from the group consisting of: fibroblasts, keratinocytes, epithelial cells, endothelial cells, glial cells, neural cells, formed elements of the blood, hepatocytes and precursors thereof.

98. A method of Claim 97 wherein the glucagon-like peptide 1 related peptide is a glucan-like peptide 1 derivative selected from the group consisting of GLP-1(7-37), GLP-1(7-36), GLP-1(7-35) GLP-1(7-34) and other truncated carboxy-terminal amidated derivatives and derivatives of GLP-1 which have amino acid substitutions, deletions, additions or other alterations (e.g., addition of a non-amino acid component) which result in biological activity or stability in the blood which is substantially the same as that of a truncated GLP-1 derivative or enhanced biological activity or stability in the blood.

99. A method of providing a glucagon-like peptide 1 related peptide in an effective amount to a mammal, comprising the steps of:

a) obtaining a source of primary cells from the mammal;

b) producing a population of secondary cells from the primary cells provided in (a);

c) transfecting secondary cells produced in (b) with a DNA construct comprising exogenous DNA encoding a glucagon-like peptide 1 related peptide and additional DNA sequences sufficient for expression of the exogenous DNA in the primary cells, thereby producing transfected secondary cells which express the exogenous DNA encoding glucagon-like peptide;

d) culturing a transfected secondary cell which expresses the exogenous DNA encoding glucagon-like peptide produced in (c), under conditions appropriate for propagating the transfected secondary cell which expresses the exogenous DNA encoding a glucagon-like peptide 1 related peptide, thereby producing a clonal cell strain of transfected secondary cells from the transfected secondary cell;

e) culturing the clonal cell strain of transfected secondary cells produced in (c) under conditions appropriate for and sufficient time for the clonal cell strain of transfected secondary cells to undergo a sufficient number of doublings to provide a sufficient number of transfected secondary cells to produce an effective amount of a glucagon-like peptide 1 related peptide; and

f) introducing transfected secondary cells produced in (e) into the mammal in sufficient number to produce an effective amount of a glucagon-like peptide 1 related peptide.

100. A method of Claim 99 wherein the glucagon-like peptide 1 related peptide is a glucan-like peptide 1 derivative selected from the group consisting of GLP-1(7-37), GLP-1(7-36), GLP-1(7-35) GLP-1(7-34) and other truncated carboxy-terminal amidated derivatives and derivatives of GLP-1 which have amino acid substitutions, deletions, additions or other alterations (e.g., addition of a non-amino acid component) which result in biological activity or stability in the blood which is substantially the same as that of a truncated GLP-1 derivative or enhanced biological activity or stability in the blood.

101. A method of producing a glucagon-like peptide 1 related peptide in an effective amount to a mammal, comprising the steps of:

a) obtaining a source of primary cells from the mammal;

b) transfecting primary cells obtained in (a) with exogenous DNA encoding a glucagon-like peptide 1 related peptide and operatively linked to DNA sequences of non-retroviral origin sufficient for expression of the exogenous DNA in transfected secondary cells, thereby producing a mixture of primary cells which includes

transfected primary cells which express the exogenous DNA encoding a glucagon-like peptide 1 related peptide;

c) culturing the product of (b) under conditions appropriate for propagation of transfected primary cells which express the exogenous DNA encoding a glucagon-like peptide 1 related peptide, thereby producing a heterogenous cell strain of transfected secondary cells of vertebrate origin which express the exogenous DNA encoding a glucagon-like peptide 1 related peptide; and

d) introducing transfected secondary cells produced in (c) into the mammal in sufficient number to produce an effective amount of a glucagon-like peptide 1 related peptide in the mammal.

102. A method of Claim 101 wherein the glucagon-like peptide 1 related peptide is a glucan-like peptide 1 derivative selected from the group consisting of GLP-1(7-37), GLP-1(7-36), GLP-1(7-35) GLP-1(7-34) and other truncated carboxy-terminal amidated derivatives and derivatives of GLP-1 which have amino acid substitutions, deletions, additions or other alterations (e.g., addition of a non-amino acid component) which result in biological activity or stability in the blood which is substantially the same as that of a truncated GLP-1 derivative or enhanced biological activity or stability in the blood.

103. A method of providing a glucagon-like peptide 1 related peptide in an effective amount to a mammal, comprising the steps of:

a) obtaining a source of primary cells from the mammal;

b) producing a population of secondary cells from the primary cells provided in (a);

c) transfecting secondary cells produced in (b) with exogenous DNA encoding a glucagon-like peptide 1 related peptide and operatively linked to DNA sequences of non-retroviral origin sufficient for expression of the exogenous DNA in transfected secondary cells, thereby producing a mixture of secondary cells which includes transfected secondary cells which express the exogenous DNA encoding a glucagon-like peptide 1 related peptide;

d) culturing the product of (c) under conditions appropriate for propagation of transfected secondary cells which express the exogenous DNA encoding a glucagon-like peptide 1 related peptide, thereby producing a heterogenous cell strain of transfected secondary cells of vertebrate origin which express the exogenous DNA encoding glucagon-like peptide; and

e) introducing transfected secondary cells produced in (c) into the mammal in sufficient number to produce an effective amount of a glucagon-like peptide 1 related peptide in the mammal.

104. A method of Claim 103 wherein the glucagon-like peptide 1 related peptide is a glucan-like peptide 1 derivative selected from the group consisting of GLP-1(7-37), GLP-1(7-36), GLP-1(7-35) GLP-1(7-34) and other truncated carboxy-terminal amidated derivatives and derivatives of GLP-1 which have amino acid substitutions, deletions, additions or other alterations (e.g., addition of a non-amino acid component) which result in biological activity or stability in the blood which is substantially the same as that of a truncated GLP-1 derivative or enhanced biological activity or stability in the blood.

105. A method of providing erythropoietin in an effective amount to a mammal, comprising introducing into the mammal a barrier device containing:

a) transfected primary cells expressing exogenous DNA encoding erythropoietin,

b) transfected secondary cells expressing exogenous DNA encoding erythropoietin,

c) or both a) and b),

wherein the barrier device is made of a material which permits passage of erythropoietin into the circulation or tissues of the mammal and prevents contact between the immune system of the mammal and the transfected cells contained within the barrier device to a sufficient extent to prevent a deleterious immune response by the mammal.

106. A method of providing erythropoietin in an effective amount to a mammal, comprising introducing into the mammal a DNA construct comprising exogenous DNA encoding erythropoietin and regulatory sequences sufficient for expression of erythropoietin in cells of the mammal, wherein the DNA construct is taken up by cells of the mammal and is expressed therein.

107. The method of Claim 106 wherein the DNA construct is introduced into the mammal by direct injection into muscle.

## Abstract of the Invention

The present invention relates to transfected primary and secondary somatic cells of vertebrate origin, particularly mammalian origin, transfected with exogenous genetic material (DNA) which encodes erythropoietin or an insulinotropin [e.g., derivatives of glucagon-like peptide 1(GLP-1)], methods by which primary and secondary cells are transfected to include exogenous genetic material encoding erythropoietin or an insulinotropin, methods of producing clonal cell strains or heterogenous cell strains which express eruthropoietin or an insulinotropin, methods of gene therapy in which the transfected primary or secondary cells are used, and methods of producing antibodies using the transfected primary or secondary cells.

The present invention includes primary and secondary somatic cells, such as fibroblasts, keratinocytes, epithelial cells, endothelial cells, glial cells, neural cells, formed elements of the blood, muscle cells, other somatic cells which can be cultured and somatic cell precursors, which have been transfected with exogenous DNA encoding EPO or an insulinotropin, which is stably integrated into their genomes or is expressed in the cells episomally.

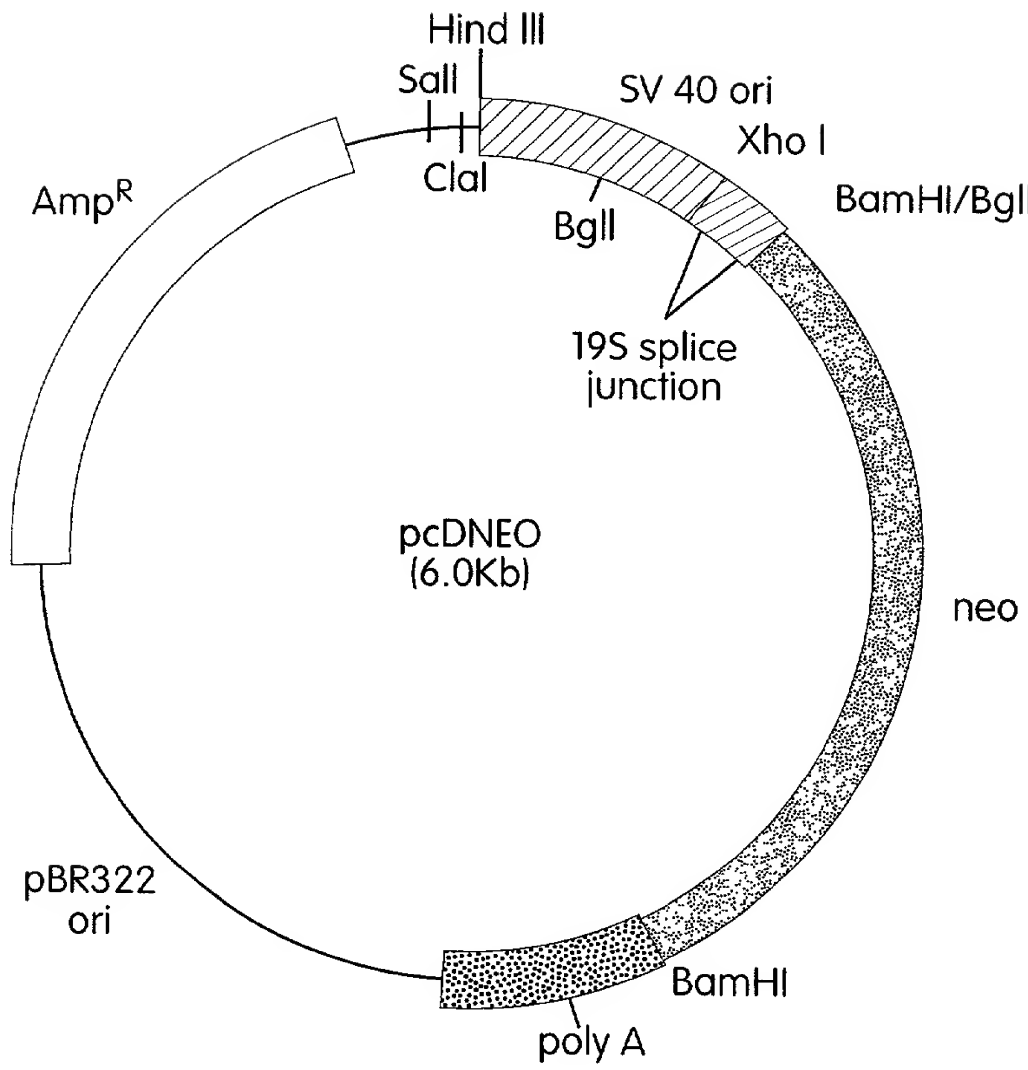
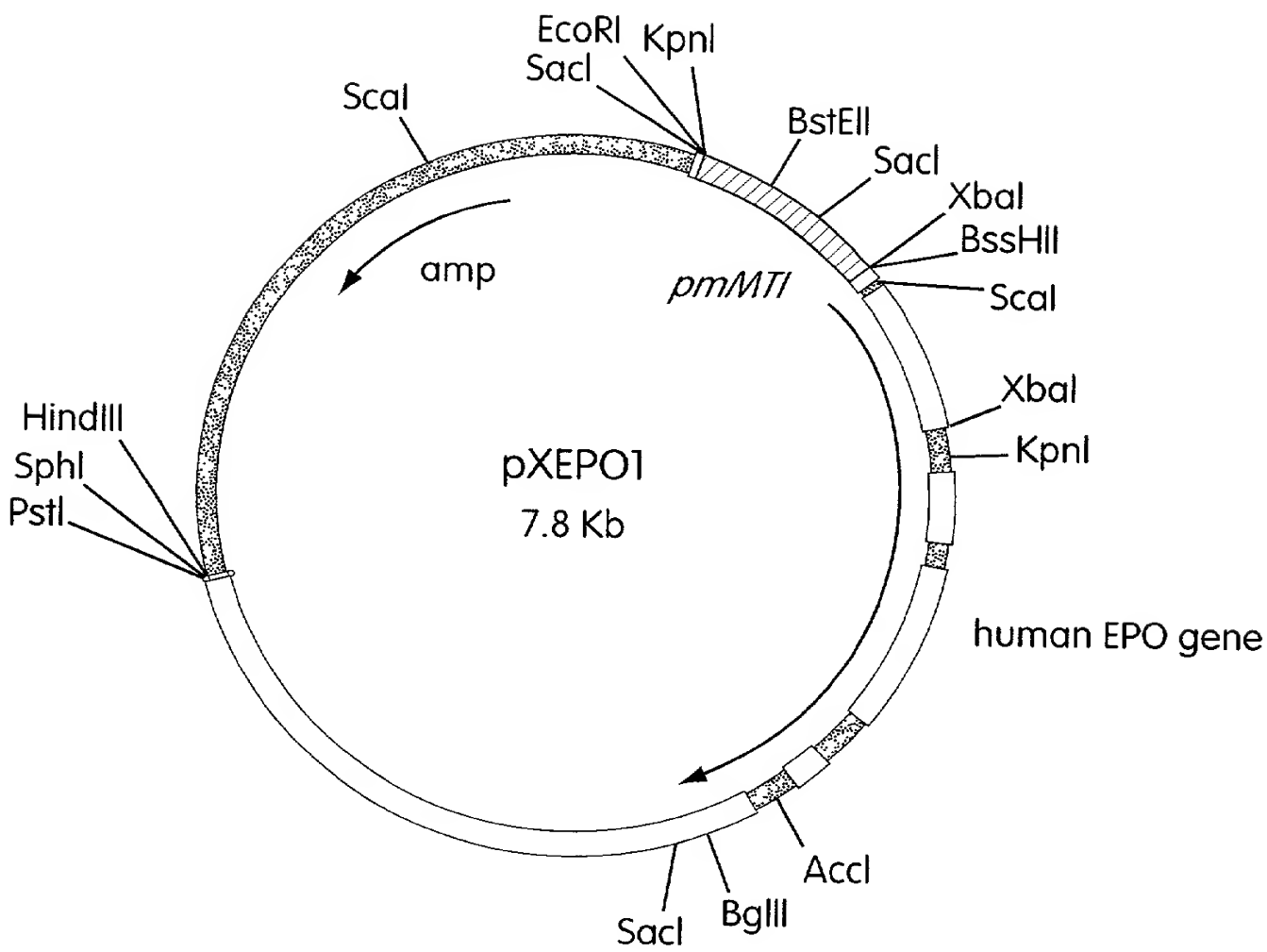

Hind III
SalI
SV 40 ori
Xho I
ClaI
Amp^R
BglI
BamHI/BglII
19S splice
junction
pcDNEO
(6.0Kb)
neo
pBR322
ori
BamHI
poly A

Fig. 1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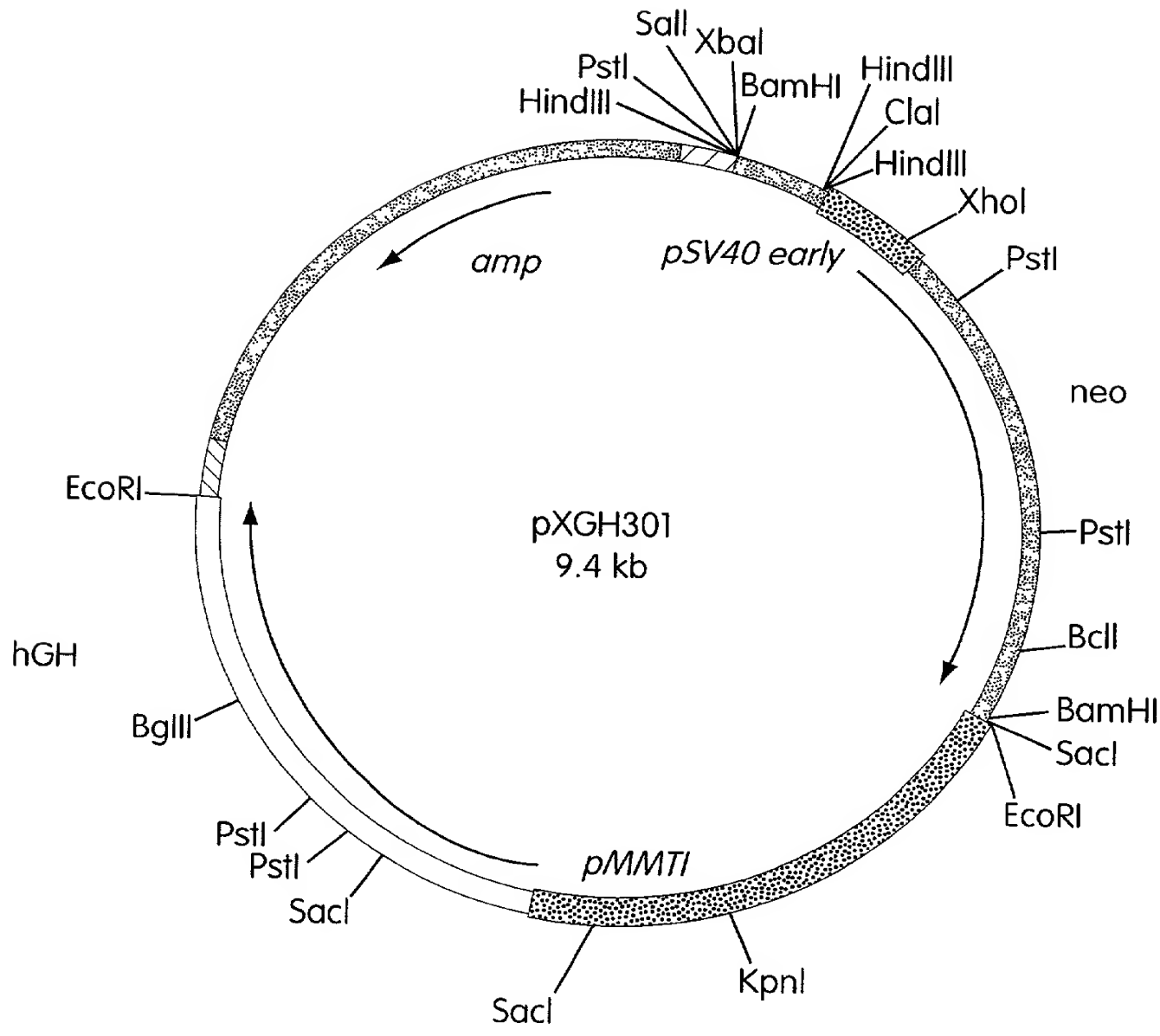
KpnI
SacI
ScaI
BstEII
SacI
XbaI
BssHII
ScaI
amp
pmMTI
XbaI
KpnI
HindIII
SphI
PstI
pXEPO1
7.8 Kb
human EPO gene
AccI
BglII
SacI

Fig. 2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XbaI
PstI
BamHI
HindIII
HindIII
ClaI
HindIII
XhoI
PstI
amp
pSV40 early
neo
EcoRI
pXGH301
9.4 kb
PstI
BclI
hGH
BamHI
BglII
SacI
EcoRI
PstI
PstI
pMMTI
SacI
SacI
KpnI

Fig. 3

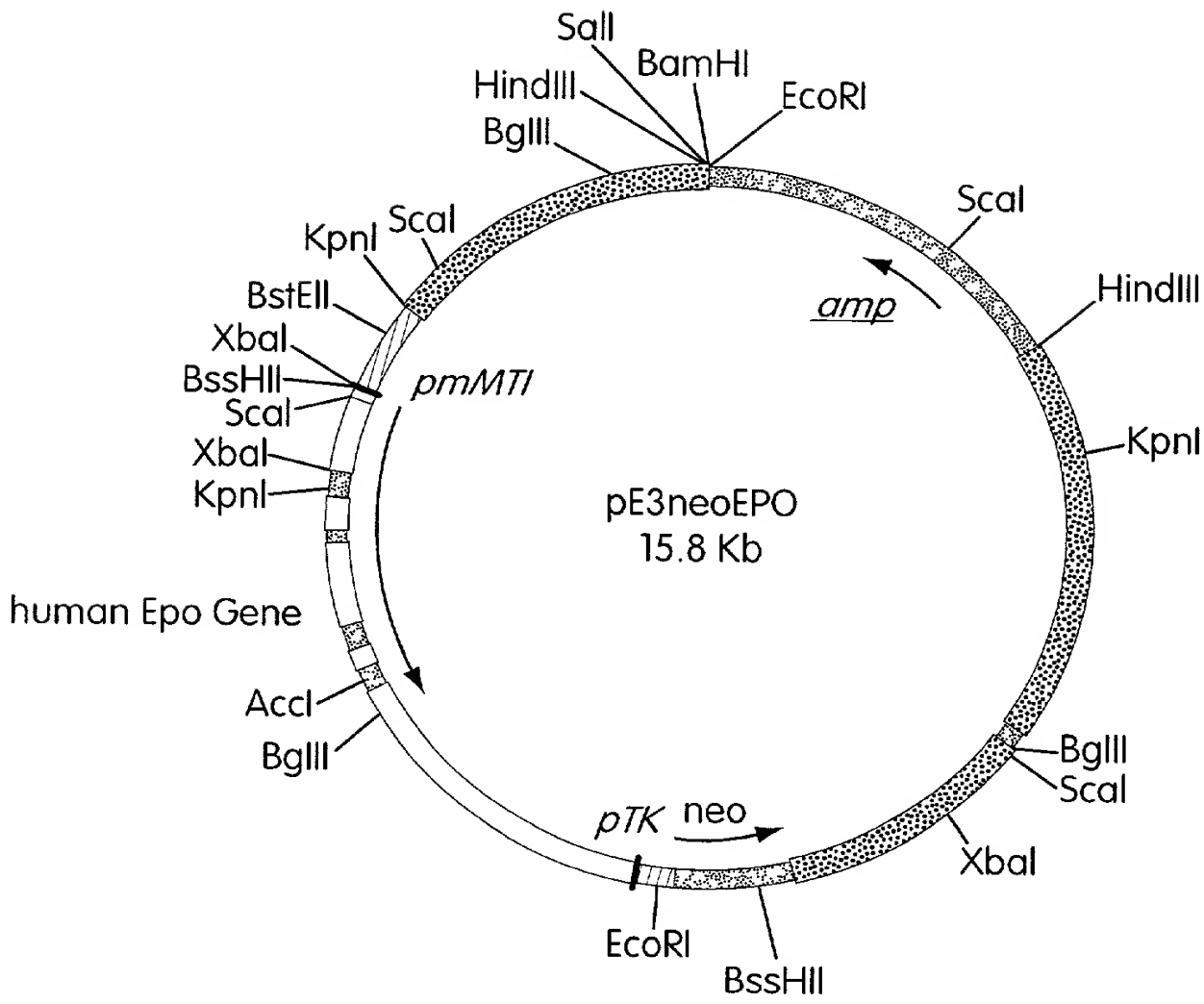
SalI
BamHI
HindIII
EcoRI
BglII
ScaI
KpnI
ScaI
BstEII
XbaI
BssHII
ScaI
XbaI
KpnI
pmMTI
amp
HindIII
KpnI
pE3neoEPO
15.8 Kb
human Epo Gene
AccI
BglII
BglII
ScaI
pTK
neo
XbaI
EcoRI
BssHII

Fig. 4

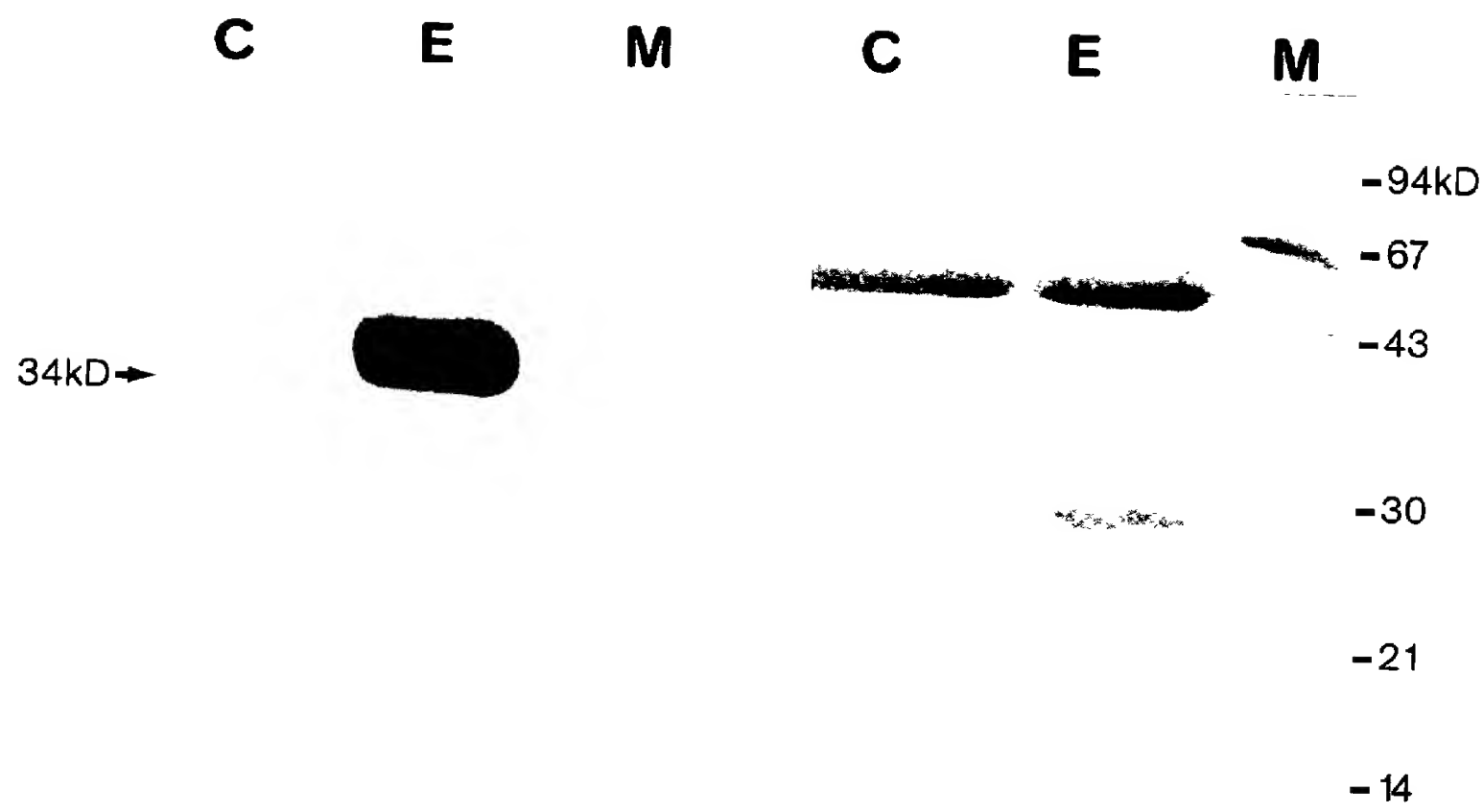


Fig. 5A

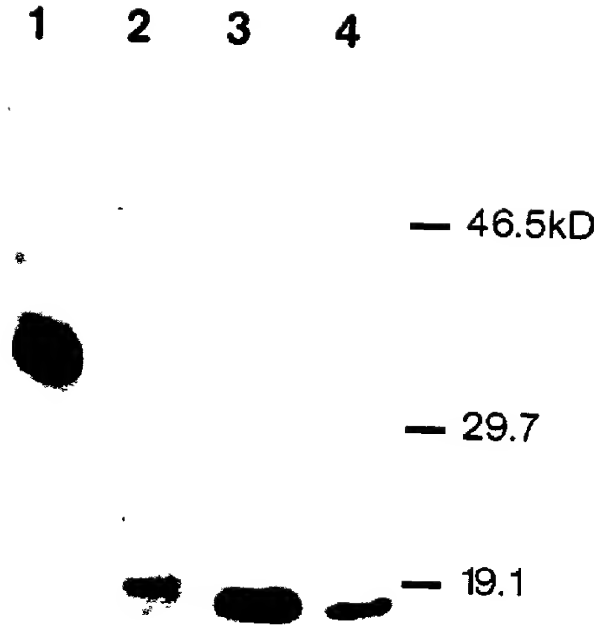


Fig. 5B

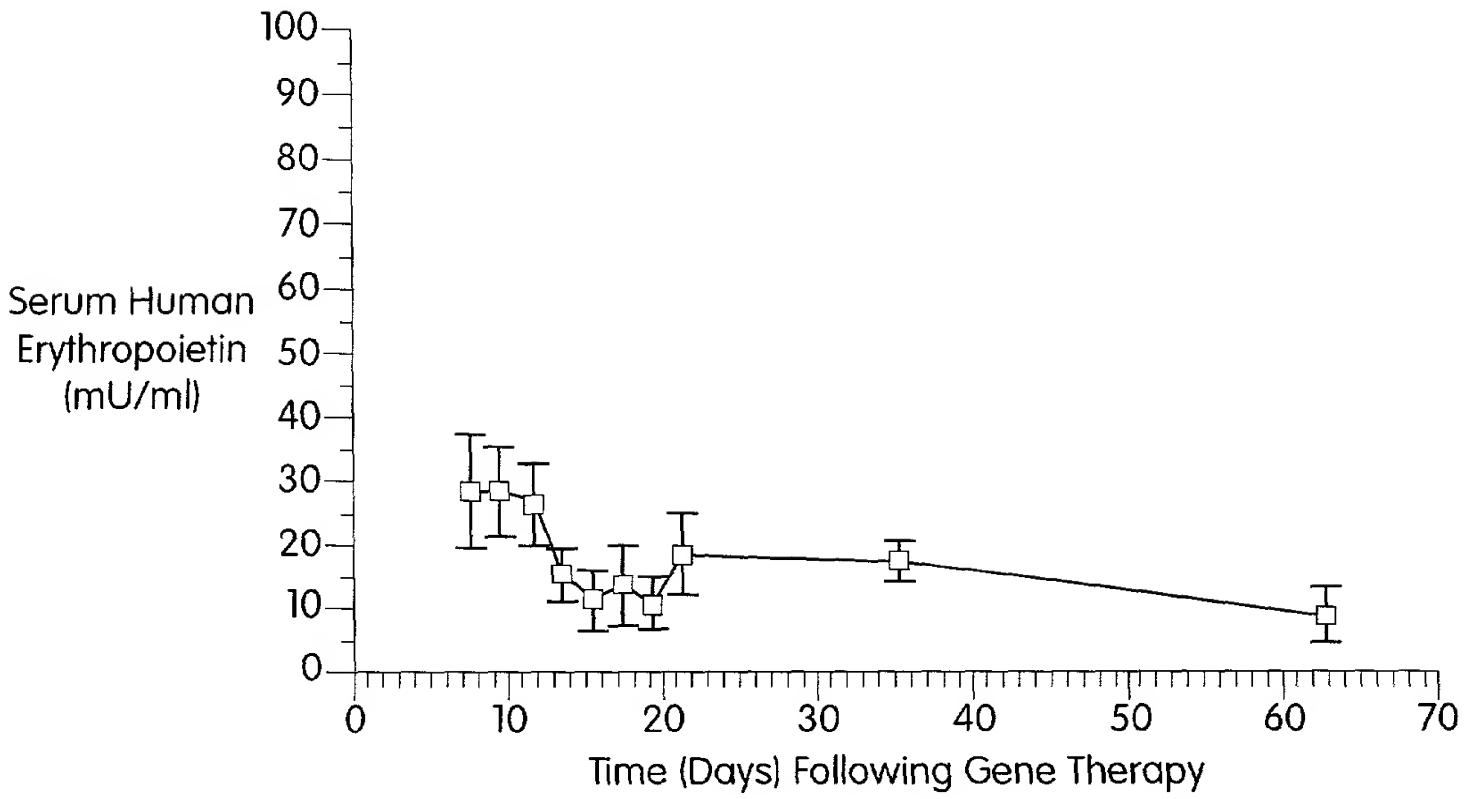
Serum Human Erythropoietin (mU/ml)
100
90
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
70
Time (Days) Following Gene Therapy

Fig. 6A

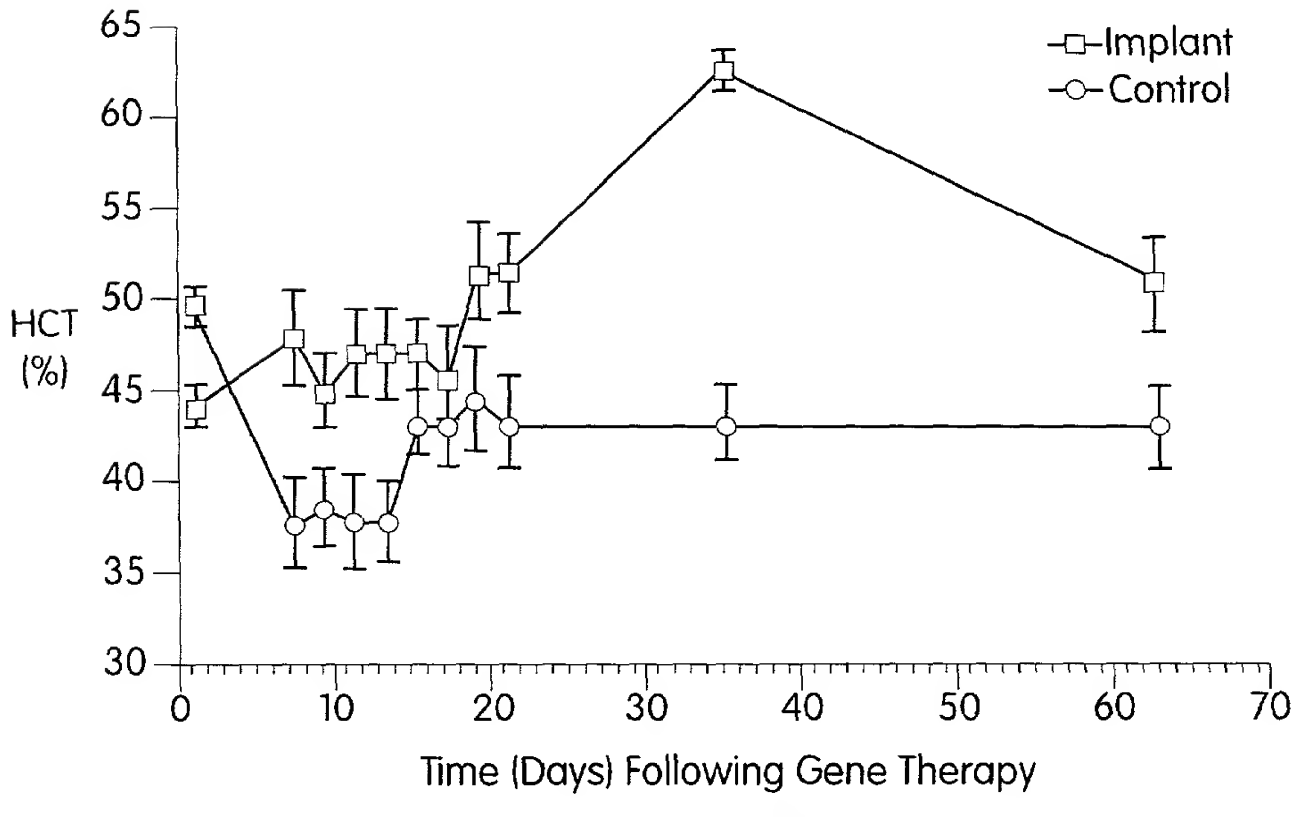
HCT (%)
65
60
55
50
45
40
35
30
0
10
20
30
40
50
60
70
Time (Days) Following Gene Therapy
Implant
Control

Fig. 6B

DOCKET NO. TKT91-01A

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

## Declaration for Patent Application

As a named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated next to my name;

I believe I am the original, first and sole inventor (if only one name is listed) or an original, first and joint inventor (if plural names are listed in the signatory page(s) commencing at page 3 hereof) of the subject matter which is claimed and for which a patent is sought on the invention entitled

IN VIVO PRODUCTION AND DELIVERY OF ERYTHROPOIETIN OR INSULINOTROPIN FOR GENE THERAPY

the specification of which (check one)

/__/ is attached hereto.

/__/ was filed on _____ as Application Serial No._____ and was amended on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is known by me to be material to patentability as defined in 37 C.F.R. §1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

### Prior Foreign Application(s)

| (Number) | (Country) | (Day/Month/Year filed) | Priority Claimed Yes | Priority Claimed No |
|---|---|---|---|---|
| | | | /__/ | /__/ |
| | | | /__/ | /__/ |
| | | | /__/ | /__/ |

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose information known by me to be material to patentability as defined in 37 C.F.R. §1.56 which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

| | | |
|---|---|---|
| 07/787,840 | November 5, 1991 | Pending |
| (Application Serial No.) | (Filing date) | (Status, patented, pending, abandoned) |
| (Application Serial No.) | (Filing date) | (Status, patented, pending, abandoned) |

As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

I also hereby grant additional Powers of Attorney to the following attorney(s) and/or agent(s) to file and prosecute an international application under the Patent Cooperation Treaty based upon the above-identified application, including a power to meet all designated office requirements for designated states.

| | |
|---|---|
| David E. Brook | Registration No. 22,592 |
| James M. Smith | Registration No. 28,043 |
| Leo R. Reynolds | Registration No. 20,884 |
| Richard A. Wise | Registration No. 18,041 |
| Patricia Granahan | Registration No. 32,227 |
| Mary Lou Wakimura | Registration No. 31,804 |
| Thomas O. Hoover | Registration No. 32,470 |
| Alice O. Carroll | Registration No. 33,542 |

all of Hamilton, Brook, Smith and Reynolds, P.C., Two Militia Drive, Lexington, Massachusetts 02173;

and

_____

Send correspondence to: Patricia Granahan, Esq.
HAMILTON, BROOK, SMITH & REYNOLDS, P.C.
Two Militia Drive, Lexington, MA 02173

Direct telephone calls to: Patricia Granahan, Esq.

(617) 861-6240

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full name of sole or first inventor: Richard F Selden

Inventor's Signature _____ Date _____

Residence: 106 Bristol Road, Wellesley, MA 02181

Citizenship: U.S.A.

Post Office Address: same as above

---

Full name of second joint inventor, if any: Douglas Treco

Second Inventor's Signature _____ Date _____

Residence: 87 Brantwood Road, Arlington, MA 02174

Citizenship: U.S.A.

Post Office Address: same as above

---

Full name of third joint inventor, if any: Michael W. Heartlein

Third Inventor's Signature _____ Date _____

Residence: 167 Reed Farm Road, Boxborough, MA 01719

Citizenship: U.S.A.

Post Office Address: same as above

---

Full name of fourth joint inventor, if any _____

Fourth Inventor's Signature _____ Date _____

Residence _____

Citizenship _____

Post Office Address _____

---

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PATENT
ATTORNEY DOCKET NO. 50010/007001

Certificate of Mailing: Date of Deposit: April 17, 1997

I hereby certify under 37 CFR 1.8(a) that this correspondence is being deposited with the United States Postal Service as **first class mail** with sufficient postage on the date indicated above and is addressed to the Assistant Commissioner of Patents and Trademarks, Washington, D.C. 20231.

Matthew G. Doherty
Printed name of person mailing correspondence

Matthew G. Doherty
Signature of person mailing correspondence

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

| | | | | |
|---|---|---|---|---|
| Applicant | : | Richard F Selden et al. | Art Unit | : 1805 |
| Serial No. | : | 08/334,455 | Examiner | : Schwartzman |
| Filed | : | November 4, 1994 | | |
| Title | : | *IN VIVO* PRODUCTION AND DELIVERY OF ERYTHROPOIETIN OR INSULINOTROPIN FOR GENE THERAPY | | |

Assistant Commissioner of Patents and Trademarks
Washington, DC 20231

REVOCATION AND NEW POWER OF ATTORNEY

Under 37 CFR §3.73(b) Transkaryotic Therapies, Inc., a corporation, certifies that it is the assignee of 100% of the right, title and interest in the patent application identified above by virtue of:

☒ An assignment from the inventors of U.S. Serial No. 07/911,533, of which the patent application identified above is a file wrapper continuation application under 37 CFR §1.62. The assignment was recorded in the Patent and Trademark Office at Reel 6251, Frame 0898 on September 16, 1992.

The undersigned has reviewed all the documents in the chain of title of the patent application identified above and, to the best of undersigned's knowledge and belief, title is in the assignee identified above.

The undersigned, whose title is supplied below, is empowered to act on behalf of the assignee.

The undersigned, acting on behalf of the assignee, hereby revokes all powers of attorney previously granted in the above-identified application and appoints:

Paul T. Clark, Reg. No. 30,162
Karen Lech Elbing, Reg. No. 35,238
Clark & Elbing LLP
176 Federal Street
Boston, MA 02110-2214

with full power of substitution and revocation, to prosecute the above-identified application and to transact all business in the United States Patent and Trademark Office connected therewith.

All correspondence regarding the application should be sent to Paul T. Clark, Clark & Elbing LLP, 176 Federal Street, Boston, MA 02110-2214, (617) 428-0200.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

Respectfully submitted,

Date: 7/11/97

Richard F Selden, M.D., Ph.D.
President and CEO
Transkaryotic Therapies, Inc.

TKT911AF.SDC
CSE10
PG/CSE/jwb
03/01/95

PATENT APPLICATION
Docket No.: TKT91-01AF

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Richard F Selden, Douglas Treco and Michael W. Heartlein

Serial No.: 08/334,455 Group Art Unit: 1805

Filed: November 4, 1994 Examiner: R. Hodges

For: IN VIVO PRODUCTION AND DELIVERY OF ERYTHROPOIETIN OR INSULINOTROPIN FOR GENE THERAPY

CERTIFICATE OF MAILING

I hereby certify that this correspondence is being deposited with the United States Postal Service with sufficient postage as First Class Mail in an envelope addressed to Honorable Commissioner of Patents and Trademarks, Washington D.C. 20231

on 3/2/95 Judy Breen
Date Signature

Judy Breen
Typed or printed name of person signing certificate

TRANSMITTAL OF SUBSTITUTE DECLARATIONS/POWER OF ATTORNEY

The Honorable Commissioner of
Patents and Trademarks
Washington, DC 20231

COPY

Attn: Application Division

Sir:

Enclosed herewith is an executed Substitute Declarations/Power of Attorney for filing in the captioned application. The Substitute Declarations correct the claim for priority to Serial No. 07/789,188, inadvertently omitted from the Declarations filed September 1, 1992.

Please charge Applicant's Attorney's Deposit Account No. 08-0380 for any additional fees that may be due in this matter. Two duplicate copies are enclosed for that purpose.

Respectfully submitted,

Carolyn S. Elmore
Patent Agent
Registration No. 37,567
Telephone: (617) 861-6240

Lexington, MA
Date: 3/2/95

DOCKET NO. **TKT91-01A**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Substitute
Declaration for Patent Application

As a named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated next to my name;

I believe I am the original, first and sole inventor (if only one name is listed) or an original, first and joint inventor (if plural names are listed in the signatory page(s) commencing at page 3 hereof) of the subject matter which is claimed and for which a patent is sought on the invention entitled

IN VIVO PRODUCTION AND DELIVERY OF ERYTHROPOIETIN OR INSULINOTROPIN FOR GENE THERAPY

the specification of which (check one)

[ ] is attached hereto.

[X] was filed on July 10, 1992 as Application Serial No 07/911,533 and was amended on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is known by me to be material to patentability as defined in 37 C.F.R. §1.56.

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Prior Foreign Application(s)

| (Number) | (Country) | (Day/Month/Year filed) | Priority Claimed [ ] Yes [ ] No |
|---|---|---|---|
| | | | [ ] Yes [ ] No |
| | | | [ ] Yes [ ] No |

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| (Application Serial No.) | (Filing date) | (Status, patented, pending, abandoned) |
| 07/789,188 | November 5, 1991 | Abandoned |
| (Application Serial No.) | (Filing date) | (Status, patented, pending, abandoned) |

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| Leo R. Reynolds | Registration No. 20,884 |
| Richard A. Wise | Registration No. 18,041 |
| Patricia Granahan | Registration No. 32,227 |
| Mary Lou Wakimura | Registration No. 31,804 |
| Thomas O. Hoover | Registration No. 32,470 |
| Alice O. Carroll | Registration No. 33,542 |

all of Hamilton, Brook, Smith and Reynolds, P.C., Two Militia Drive, Lexington, Massachusetts 02173;

and

_____

Send correspondence to: Patricia Granahan

HAMILITON, BROOK, SMITH & REYNOLDS, P.C.

Two Militia Drive, Lexington, Massachusetts 02173

Direct telephone calls to: Patricia Granahan

(617) 861-6240

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---

Full name of sole or first inventor: Richard F Selden

Inventor's Signature: Richard F Selden Date: 013195

Residence: 106 Bristol Road
Wellesley, Massachusetts 02181

Citizenship: U.S.A.

Post Office Address: Same as above

---

Full name of second joint inventor, if any: Douglas Treco

Second Inventor's Signature: Douglas Treco Date: 2/3/95

Residence: 87 Brantwood Road
Arlington, Massachusetts 02174

Citizenship: U.S.A.

Post Office Address: Same as above

---

Full name of third joint inventor, if any: Michael W. Heartlein

Third Inventor's Signature: Michael W. Heartlein Date: 2/7/95

Residence: 167 Reed Farm Road
Boxborough, Massachusetts 01719

Citizenship: U.S.A.

Post Office Address: Same as above

---

Full name of fourth joint inventor, if any:

Fourth Inventor's Signature: Date:

Residence:

Citizenship:

Post Office Address:

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PATENT
ATTORNEY DOCKET NO. 50010/007006

Certificate of Mailing

Date of Deposit: June 8, 1999 Label Number: EL356089537US

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Sandra E. Marxen — Sandra E. Marxen
Printed name of person mailing correspondence — Signature of person mailing correspondence

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Richard F Selden *et al.* Art Unit:

Serial No.: Not Yet Assigned Examiner:

Filed: Herewith

Title: *IN VIVO* PRODUCTION AND DELIVERY OF ERYTHROPOIETIN OR INSULINOTROPIN FOR GENE THERAPY

BOX PATENT APPLICATION
Assistant Commissioner of Patents
Washington, DC 20231

TRANSMITTAL OF FORMAL DRAWINGS TO OFFICIAL DRAFTSPERSON

Enclosed please find seven sheets of formal drawings consisting of FIGs. 1 - 6B.

If there are any other charges, or any credits, please apply them to Deposit Account No. 03-2095.

Respectfully submitted,

Date: June 8, 1999

Susan M. Michaud

Paul T. Clark
Reg. No. 30,162

Susan M. Michaud
Reg. No. 42,885

Clark & Elbing LLP
176 Federal Street
Boston, MA 02110
Telephone: 617-428-0200
Facsimile: 617-428-7045

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